ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN PREGNANT WOMEN WITH BAD OBSTETRIC HISTORY

Dissertation submitted in partial fulfillment of the Requirement for the award of the Degree of

M.D.MICROBIOLOGY

(BRANCH IV)

DEPARTMENT OF MICROBIOLOGY
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CERTIFICATE

This is to certify that the Dissertation **"Association of Antinuclear Antibodies in Pregnant Women with Bad Obstetric History"** presented herein by **Dr.A.Anupriya** is an original work done in the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D. (Branch IV) Microbiology under my guidance and supervision during the academic period of 2010 - 2013.

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This is to certify that the dissertation entitled, **‘‘Association of Antinuclear Antibodies in Pregnant Women with Bad Obstetric History”** by **Dr.A.Anupriya,** Post graduate in Microbiology (2010-2013), is a bonafide research work carried out under our direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in **April 2013.**

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The dissertation is submitted to The Tamilnadu Dr. M.G.R.Medical University towards the partial fulfilment of requirements for the award of M.D. Degree (Branch IV) in Microbiology.

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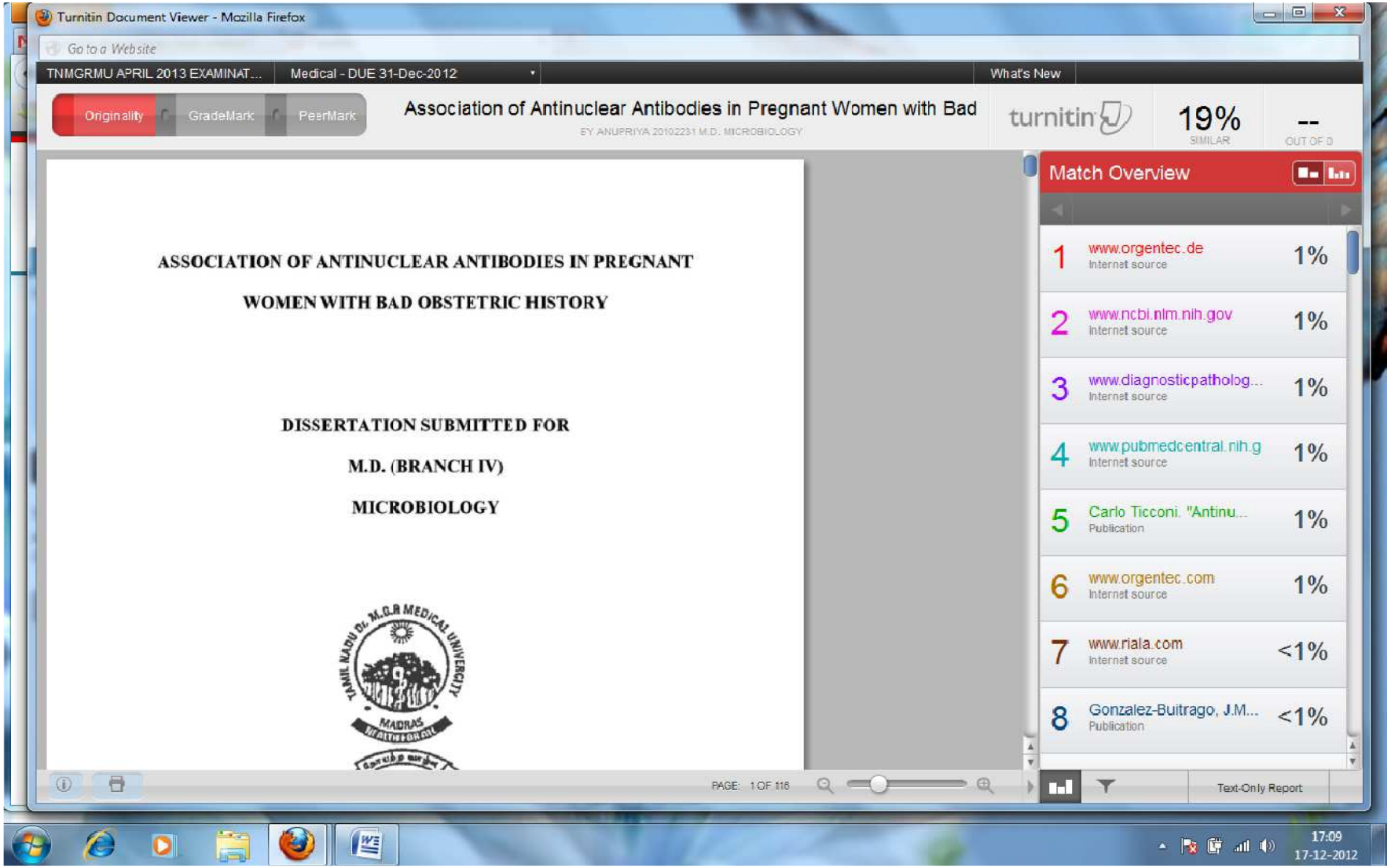
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Introduction

1.INTRODUCTION

Immune status, individual response to disease and types of antibodies produced are known to vary from person to person, place to place and from population to population. A broad spectrum of specific auto antibodies which are associated with specific rheumatic diseases, as noted in Western literature, has been taken as a reference standard all over the world[1]. There is neither research work nor any data correlating the auto antibodies and their antinuclear antibody (ANA) patterns with the immune profile in the Indian population to date.

About12-15% of clinically recognizable pregnancies result in abortion. "Abortion is defined as the loss of a fetus or embryo weighing ≤ 500g, which would normally be at 20-22 complete weeks of gestation as per World Health Organisation". (WHO 1977).

No valid estimate of incidence of abortion is known, because denominator variable (=individuals at risk) not clear[2]. Around 1% of fertile couples have recurrent pregnancy loss of which, in 50% of patients the underlying cause remains unknown. Of the known causes, autoimmune factors contribute to 5-10% of recurrent pregnancy loss.

Autoimmune factors have been recognised as key factors in recurrent pregnancy loss, even in women with no clinically diagnosed autoimmune disease.

The term Bad Obstetric History(BOH)[3]," is applied to pregnant women where her present obstetric outcome is likely to be affected adversely by the nature of previous obstetric disaster". In obstetrics, any complicating factor, known or unknown is likely to recur and if it recurs in two consecutive pregnancies, the chance of its recurrence in third pregnancy is highly probable

1.2 Etiology of recurrent pregnancy loss[3]

The causes of recurrent fetal wastage are complex and most often obscure. More than one factor may operate in a case. Factors may be recurrent or non-recurrent.

1.2.1First trimester abortion

1.**Genetic causes**

It accounts for 3-5% of fetal wastage. Parental chromosomal abnormalities is a prove cause of recurrent abortion. The most common abnormality is a balanced translocation. Risk of miscarriage in couples with a balanced translocation is more than 25%.However the chance of successful pregnancy even without treatment is 40-50%.

2. Endocrine and metabolic

- Poorly controlled diabetic patients may have an increased incidence of early pregnancy failure
- Presence of thyroid autoantibodies is often associated with increased risk

- Luteal phase defect with less production of progesterone, interfers with endometrial maturation
- Hyper-secretion of Luteinising hormone as seen in PCOS cases is associated with sub-fertility and higher miscarriages.

3. **Infection**

Infections of genital tract may be responsible for sporadic spontaneous abortions.Trans-placental fetal infection can occur with most microorganisms.

4.Inherited Thrombophilia

This causes both early and late miscarriages due to intravascular thrombosis.Protein C resistance and factor V Leiden mutation is the most common cause. Hyperhomocystinemia is also a factor for recurrent miscarriages.

5.Unexplained

In the majority, the cause remains unknown.

1.2.2 Second trimester abortion

1.**Anatomic abnormalities**

These are responsible for 10-15% of recurrent pregnancy loss.The causes may be congenital or acquired.

- Congenital anomalies may be due to defect in the mullerian duct fusion or resorption (eg; unicornuate, bicornuate, septate or double uterus)

- Acquired anomalies are intrauterine adhesions, uterine fibroids, endometriosis and cervical incompetence

2.Chronic Maternal illness

This includes uncontrolled diabetes, atherosclerosis, haemoglobinopathies, chronic renal disease, inflammatory bowel disease.

3.Infection

Syphilis, toxoplasmosis, Listeriosis may be responsible in some cases.

1.3 Immune factors

Immunological association of mother and the fetus is double-directional which is decided by presentation of antigen by the fetus and its recognition and reaction by the maternal immune system.Balanced immune response is mandatory to sustain pregnancy and any imbalance, can lead to early wastage of conception.

Autoantibodies and auto-reactive helper T cells(Th-2 cells) induces self-reactivity which may lead to tissue damage that results in non-organ specific disease like Systemic Lupus Erythematosus or organ specific diseases like Hashimoto's thyroiditis, Addison's disease, type I diabetes mellitus.

The immunological causes for abortions, can be divided to autoimmune factors and alloimmune factors.

1.3.1Autoimmune factors

Presence of autoantibodies causes rejection of early pregnancy in 30% of women[5].Antibodies responsible are: Antinuclear antibodies and Antiphospholipid antibodies: lupus anticoagulant, anticardiolipin antibodies. These women demonstrate a tendency to miscarriage at progressively lower gestational ages. Placental vascular atherosis, intervillus thrombosis, and decidual vasculopathy with fibrinoid necrosis are the immediate pathology for fetal loss.Pregnancy complications include miscarriages, intra-uterine growth retardation, pre-eclamptic toxaemia, still birth, pre-term labour and placental abruption.

1.3.2 Alloimmune factors

There is failure of maternal recognition of trophoblast lymphocyte cross-reactive antigen(TLX).Consequently there is lack of production of blocking antibodies by the mother.This is due to sharing of Human Leucocyte Antigen between partners[3].

1.3.3 Autoimmunity- role in RPL

Any disturbance in humoral or cellular response or function causes immune reaction against self components, which are manifested as signs and symptoms in autoimmune disorders.Genetic or environmental or other unknown factors triggers, a break in self tolerance[6].

Pregnancy is confronted with number of self and foreign antigens that modulate the immune system of the mother. If immune response of

mother is altered, frequent abortions result. Autoimmune disorders are 6 to 10 times more common among women than men[7], and are most likely to have their onset during the reproductive years.

1.4.1 ANA prevalence global scenario

An estimated 9.8 million women are afflicted with one of the seven more common autoimmune diseases: lupus, scleroderma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, Sjogren's, and type 1 diabetes[8]. The prevalence estimate of SLE in the general population is approximately 40:100,000, but this condition may affect 1:1000 women during pregnancy. It is estimated that 10% of all women with lupus are diagnosed with the disease either during pregnancy or in the immediate postpartum period[9].

1.4.2 ANA prevalence in Indian Scenario

In India, the prevalence of SLE is 3.2 per 1,00,000(Medscape),the overall annual incidence being 5-100 per 100,000 populations; more than 90% of the patients are females of reproductive age[10].

It is reported that nearly half of lupus pregnancies are normal, one-fourth deliver premature babies, and one-fourh of patients suffer from recurrent miscarriages.

Autoimmune disorders are most likely to have their onset during the reproductive years. It is therefore common to encounter patients with these conditions during pregnancy. The predictive value of screening

patients for subclinical autoimmune disease has been a matter of controversy for decades.The risk of abortions is increased in some patients with autoantibodies, even before a clinical diagnosis could be made.

1.5.1 Pathophysiology of autoimmunity in women

Females are having higher CD4/CD8 ratio and higher levels of serum immunoglobulin M.Large amont of cytokines are produced by T-helper cells. Sex steroids can induce autoimmune process, because of their ability to modify immune response via their action through steroidal receptors. These sex steroidal levels can fluctuate dramatically during gestation.This hormonal difference alone is insufficient for explaining sex difference in autoimmune diseases[2].

Interest in these disorders has increased recently for two reasons.

1. Immune system is involved in normal pregnancy and abnormal immune activation may give clues regarding the adverse outcome in patients with fetal losses.

2. Recurrent abortions and its association with certain autoantibodies (the antiphospholipid antibodies) could explain the cause for poor outcomes in patients with previously unexplained complications.

Most autoimmune disorders are so uncommon that only few prospective studies exist to provide accurate information for counselling patients about the risks, outcomes, and appropriate therapies.

1.5.2 Primary and secondary Autoantibodies

The antibodies which have direct casual relationship for the development of diseases are called 1º autoantibodies and which are not involved are called 2º autoantibodies.These 2º autoantibodies are important because, they are necessary for diagnosis and classification of the clinical conditions[5].

1.5.3**Autoantibodies associated with recurrent pregnancy loss**

Different types of autoantibodies are responsible for poor gestational outcomes which includes- Antiphospholipid antibodies, anti-β 2 glycoprotein I, antibodies directed against nuclear antigens, anti-thyroglobulin antibodies, anti-laminin, antiprothrombin antibodies(aPTs), anti-sacchromyces cervisiae antibodies[6].

1.Antinuclear antibodies

These are antibodies directed against self, which binds and destroys the nucleus of mammalian cells[6]. Autoantibodies are important for protein synthesis and regulation of cell cycle, which is a factor for normal cell activity[2]. Low levels of these antibodies may be seen in normal individuals and it could also be seen in higher frequency in normal elderly population.An increase in titre is seen in patients with connective tissue disorders[4].

Antibodies directed against nuclear proteins interfere with the formation and maturation of placenta and may lead to early fetal loss.The

complex formed by histone proteins and deoxyribonucleic acid induces tissue development. Formation of antibodies against these histone proteins may lead to activation of autoimmune process in mother, but by which it leads to recurrent gestational loss is unknown.

All forms of cells of fetal origin can cross placenta, during normal gestation and reach the maternal circulation and tissues, where they remain for long time even after delivery. This is called fetal microchimerism[11].Nelson formulated that this fetal microchimerism is the key factor for the development of autoimmune disorders, particularly antinuclear antibodies.

This hypothesis is strengthened by the higher number of these cases among females, and it peaks after gestational periods; its parallel reaction of chronic graft versus host disease to some self-reactive disorders.

Effects of Antinuclear antibodies on pregnancy

Antinuclear antibodies cause inflammatory effects in the uterus thereby it creates an environment which does not allow the adhesion of embryo. Maternal immune cells misinterpret the cells of fetal origin as malignant cells and react against them. These patients with dysregulated immune system may be affected by infertility, endometrial hyperplasia, recurrent fetal wastage because higher titres of antinuclear antibodies in their circulation[12]. Combination of antiphospholipid antibodies and

antinuclear antibodies have adverse effect on the conceptual product, in contrary to anti-thyroid antibodies.Presence of this antibodies will not have any direct toxic effect on embryo, but it can lead to adverse gestational outcome.

2.Antiphospholipid antibodies

Phospholipid molecules are normal components of cell membranes and they hold the dividing cells together. These molecules are essential for development of placenta. Anti-phospholipid antibodies react with negatively charged phospholipids, which includes phosphatidyl serine and cardiolipin.

These autaoantibodies are associated with various systemic manifestations- cerebrovascular accidents,embolic manifestations of lungs, deep vein thrombosis etc[13]. In pregnancy these antibodies may react against the trophoblast resulting in sub placental clots and interfere with implantation and subsequently cause defective placentation. Necrotising decidual vascular lesions are seen in placenta. Thrombosis occurs in all trimester of pregnancy resulting in complications such as spontaneous abortions and intra-uterine growth retardation[13].

3. Antithyroid antibodies

Autoantibodies to thyroid are considered independent risk factors during gestational period.Euthyroid women with consecutive abortions have raised levels of antibodies against thyroglobulin or thyroid

peroxidase.These thyroid antibodies could be a part of generalised autoimmune reaction or direct toxic effect of antithyroid antibodies on developing embryo.Nearly one-fifth of healthy pregnant women and one-fourth of women with recurrent fetal loss have autoantibodies to thyroid[14,15].

4. Anti-neutrophils cytoplasmic antigens(ANCA)[16]

Anti Neutrophils Cytoplasmic antigens also play a role in recurrent pregnancy loss. Primed neutrophils adhere to susceptible endothelium and ANCA antibodies interact with the ANCA antigens, which results in neutrophil activation. The ANCA-activated neutrophils release factors thereby damaging the endothelium and activating alternative complement pathway with the generation of the powerful neutrophil chemoattractant complement factor 5a.

Complement factor 5a and the neutrophil complement factor 5a receptor may compose an amplification loop for ANCA-mediated neutrophil activation.[16] Complement mediated activation amplifies neutrophil influx and this activation result in severe necrotizing inflammation of the placenta and blood vessel resulting in placental infarction and its consequences.

Autoantibodies are involved both in disease pathogenesis, and diagnosis.Low titre autoantibodies are present in every 1 in 4 persons, so when diagnosing these diseases, the test used must be highly specific,

excluding false positives.Enumerous laboratory methods are in use, and newer diagnostic tools are upcoming for early diagnosis.

1.6Anti-nuclear antibodies have been categorised into two main groups

1.6.1 Autoantibodies to DNA and Histones

These are antibodies directed against single and double standed DNA protein and histone nuclear protein.Systemic Lupus Erythematosus is confirmed by the presence of antibodies against double-stranded deoxyribonucleic acid.It is a more specific marker for this disease.The drug induced lupus is diagnosed by the presence of anti-histone antibodies[17,18].

1.6.2 Autoantibodies to extractable nuclear antigens(ENA)

Antibodies may be directed against the nuclear proteins which are extracted from the nuclei by saline wash[19].Anti-smith antibodies was the first ENA discovered in 1966, which is a specific marker for lupus.Other sub-types of ENA includes ribonucleoprotein, Scl-70, Jo-1 and PM1[20,21]. The sensitivity and specificity of various autoantibodies varies depending upon the type of autoimmune disorder.

Various types of antinuclear antibodies can be identified by IFAT techniques. Samples from person with generalized self-reactive disorder may have more than one antibodies in high titres at the same point of time

1.7 Techniques for ANA detection

1.7.1 Indirect Immunofluorescence

Indirect Immunofluorescence continues to be a basic technique in autoimmunity studies. Self reacting antibodies gives specific images which are called fluorescent patterns[22].This gives clue regarding the rheumatological disorder.This method detects true positive at a higher rate, but false positive reaction in few cases are inevitable.Autoantibodies level rise when patient has active disease and is seldom detected when patient is at remission.Wide variablity of results, high inter-personal variations and semi-quantitative results are main drawbacks, yet it remains as a indispensable technique in detection of autoimmune disorders[4].

Normal titer ranges for antinuclear antibodies is age-dependent

- 1^{st} and 2^{nd} decade of life: less than 1in 20
- 2^{nd} to 6^{th} decade: less than 1 in 40
- 7^{th} decade and older: less than 1 in 80

The pattern of immunofluorescent staining should be reported along with the titer.

- In homogeneous staining, nucleus is evenly stained.It indicates antibodies to histones and deoxyribonucleoproteins.

- In speckled pattern, fine to coarse staining of nuclear material occurs.It indicates autoantibodies to Smith antigen, ribonucleoproteins etc.
- In peripheral staining pattern, nuclear lamina or pores are stained.It indicates double-stranded (ds) DNA, rheumatoid factor, and antiphospholipid.
- In nucleolar staining,nucleoli appears stained as large particles within nucleus. This pattern is associated with scleroderma and Sjogren's syndrome.
- In centromere pattern,numerous scattered particles are seen, indicative of a systemic sclerosis variant known as CREST syndrome.

95-100% of patients with Lupus shows antinuclear antibodies positivity. 65-90% in patients with Scleroderma,50-60% in Sjogren syndrome,25-30% in rheumatoid arthritis[22].Samples which gives positive result, should be confirmed by other specific methods.

1.7.2 ELISA

ELISA is also used for diagnosis of autoantibodies.Some kits contain only few autoantigens while others includes whole extracts[4].This test can be used to screen large population, because it is less labour intensive.It can be automated, but to standardise a kit, large number of

samples both from community and persons with rheumatological disorders have to be done[23].

A positive result for ANA from diagnostic tests along with signs and symptoms is diagnostic of rheumatological disorders.Since different autoantibodies are associated with one or more disease, a step-wise approach has to be followed. Screening of samples by indirect immunofluorescence or enzyme immunoassays and if the results are positive, more specific test are done to confirm clinical diagnosis.

1.7.3 Techniques used for detection of specific ANA[4]

The other techniques used for detection of specific-ANA includes Crithidia luciliae Immunofluorescence(CLIF), Farr assay (radio-labelled assay), Counter current immunoelectrophoresis(CIE), Passive haemagglutination assay(PHA), Western blot,Dot blot, Line blot Immunoassay, Multiplex Immunoassay(MIA), Flowcytometry, Antigen microarray[4].

- **CLIF assay**

CLIF assay is an immunofluorescence test, by using a trypanosome. This test has high sensitivity, and is easy to perform. There is no interference with antibodies to single-stranded DNA.It helps in identifying various antibodies classes[24].

❖ **Farr assay**

This method is also used to identify double-stranded DNA, but this method is technically challenging and involves use of radioactive-isotopes, so it is not used in most laboratories[25].

Detection of autoantibodies using gel precipitation assays

❖ **Double Immunodiffusion**

Double immunodiffusion, detects multiple antibodies at a time, is cost-effective with high specificity.This method detects true positives at a lower rate, subjective interpretation varies from person to person, need for large volume of prototype sera[4].

❖ **Counter immunoelectrophoresis**

Counter immunoelectrophoresis is also a cost-effective procedure, with high specificity, detects multiple antibodies simultaneously, but this test also detects true positives at a lower rate, need for subjective interpretation.

❖ **Passive haemagglutination**

Passive haemagglutination assay is a semiquantitative method with high specificity,but it is a time consuming procedure and needs purified antigen[4].

❖ **Western blot**

Western blot is more sensitive than, double immunodiffusion and counter immunoelectrophoresis, and has high specificity, but it is time

consuming and expensive, hence it is used in few diagnostic centres for confirmation of autoantibodies[4,22].

❖ **Line immunoassay**

Line immunoassay is a qualitative assay.This method is technically easier, with results available in 30 minutes. This method detects true positives at a higher rate and excludes false positives considerably, but distinction between certain antibodies(Sm/RNP) are difficult[29,30].

❖ **Multiplex Immunoassay**

Multiple Immunoassay,detects multiple antibodies simultaneously. Quantification of antigen-antibody reaction possible[31,32]. This method is more efficient and technically simpler than immunofluorescence, decreases the rate of false positives, removes subjective error but it is very expensive procedure[4].

❖ **Flow cytometry**

Flow cytometry gives quantitative results.It is a cost-effective procedure and fully automated.It detects true positives at a higher rate but the major drawback is, it provides single result at a time[4].

❖ **Microarray**

Microarray is a nanotechnology technique that allows simultaneous analysis of thousands of molecular parameters.Complete automation is possible, with high sensitivity and specificity but these arrays have not been commercialised[4,28].

1.7.4 Treatment options

Drugs commonly used includes aspirin, low molecular weight heparin, prednisone. Other options includes hydroxychloroquine, azathioprine. Recent research is on the usage of intravenous immunoglobulinG, paternal lymphocyte therapy, vitamin D3 analogues, Phosphodiesterase inhibitors- like pentoxiphyline[5].

Thus it is predicted that autoantibodies, represent an epiphenomenon of immune reaction leading to miscarriages.These autoantibodies may be markers of polyclonal B cell activation or an underlying T cell defect which is related to infertility and pregnancy loss.When more number of peptides and antigens are involved, the immune dysregulation becomes stronger and rejection of fetus takes place.

Thus Antinuclear antibodies are found to be associated as a result of secondary autoimmune phenomenon in recurrent spontaneous abortion, and their early diagnosis aids to give a better prognosis at an early stage of their reproductive lives, by optimising therapy regarding their clinical condition.

Aim and Objectives

2. AIM AND OBJECTIVES

- To find out the prevalence of antinuclear antibodies in pregnant women with bad obstetric history against healthy multiparous women in and around Tirunelveli district.
- To evaluate the utility of Indirect immunofluorescence using HEp-2 cells in the diagnosis of autoantibodies(Anti Nuclear Antibodies).
- To compare the efficacy of immunoflorescence test and ELISA for diagnosis and interpretation of pregnant women with bad obstetric history.

Review of literature

3. REVIEW OF LITERATURE

The autoimmune disorders tend to be common in European and American nations. This might not be true, because in third world nations, like India there are inadequate data. Moreover many cases are undiagnosed because of lack of proper diagnostic facilities. Recent studies done in near past, has found that immunologic association exist between mother and subsequent generations, that plays a role in uncomplicated gestation.If abnormal maternal immune activation takes place,during antenatal period,it leads to miscarriages.

Autoimmune diseases occur in less than five percentage of population, as a combination of genetic and environmental factors. Abnormal immune response initiated to self antigens, can cause self-reactive phenomenon, that could be picked up by serological methods Autoantibodies persist in human body for years together and circulate in blood, as a indicator of a previous self-reactive phenomenon, but their mere existence alone, is not a marker of active disease.

There are enumerous studies in literature which tried to establish a causative relationship between specific autoantibodies associated with pregnancy loss. Antinuclear antibodies (ANA) are a combination of antibodies directed against nuclear and cytoplasmic antigens. They are pivotal for protein synthesis and regulation of cell-cycle, which is a key factor for normal cell activity.Titres of less than one in eighty dilutions

may present in one-third of sexually active females, and their significance is unknown[2].(ASRM guidelines 2008).

3.1.1ANA prevalence-global scenario

Prevalence data available from 24 countries, suggest that autoimmune disease occurs within 30—50/100,000population[33], of which more than three-fourth's are females. It is the third most common cause of fatality in women below 7th decade, next to malignancies and cardio-vascular disorders.(American Autoimmune Related Disease Association 2011). Women are more commonly affected by autoimmune disorder in comparison to males (2.7 :1).

A study done by Hayashi [34] from 2181 residents of a Japanese town to find out the presence of different patterns of antinuclear antibodies. Persons with seropositivity in IFAT were tested by enzyme linked immunoassays. There they found that females were having more reactive antibodies when compared to males. Among 566 IF-ANA-positive individuals, 100 individuals were found to have 114 disease-specific autoantibodies. Of the 60 individuals, positive for disease-specific antibodies directed against nuclear antigens,one-third showed disease manifestation and more than half of the patients were asymptomatic.

Four Thousand seventy four individuals from the year 1999-2004 were screened to detect the different types of autoantibodies, by Satoh *et al*[35].Autoantibodies to nuclear antigens was assessed by indirect immunofluorescence. The ANA positivity among US population of individuals ages 12 years and older was 13.8%, and it increases as age advances,(P=0.01), and more common in females when compared to males(17.8% versus 9.6%). This ratio peaks at 5th decade. It is less common in overweight and obese individuals (age-adjusted POR 0.74) than in persons of normal weight.

C J Edwards[36] in Britain included 668 men, 666 women for a cohort study. Antibody to nuclear antigen was measured using Enzyme Immunoassay and confirmed using immunofluorescence.Seropositivity of antinuclear antibodies was present in 10.9% of males and 12.2% of females.

3.1.2 ANA prevalence in Indian population

An extrapolated prevalence of autoimmune disease in India is around 33million[33]. In developing countries like India there is repeated contacts with micro-organisms in early life which may increase the risk of development of antibodies directed against nuclear antigens in their adult life and also it showed that past infection by some specific organsims like mumps and rubella, may predispose the formation of autoantibodies in their adult life.

Ranjana *et al*[37] in Chandigarh,have done a retrospective study (1996-2006) by using IFAT reactive cases. The sample size was 36,310. The positive rate for antinuclear antibodies was 12.3%.An annual study (2006-2007), conducted on 3,435 suspected patients with rheumatological disorders, and the positive rate was 18.9%. Among them, four-fifth of the patients were adults. Female-to-male ratio of 3:1 was reported in this study.

3.1.3 ANA prevalence in South Indian population

A study conducted by Lingaraj Jeyalakshmi[38] in Bangalore to detect antinuclear antibodies and various other autoantibodies and to analyse these variations in different age groups. The total sample size was 150, with individuals from 2nd and 3rd decade in first group, 4th and 5th decade in second group and persons in 6th and above decade in third group. Subjects were selected from health camps in and around South Indian city. Antibodies to nuclear antigens were estimated by indirect immunofluorescence method. Increased seropositivity of antinuclear antibodies and other autoantibodies were noted in all three groups of individuals.

3.2 Risk factors for development of autoimmune diseases

3.2.1 ANA prevalence in accordance with age

The autoimmune diseases can be triggered by chronic stress, excessive physical activity. Persistent, low level inflammation paves the

way, for chronic autoimmune diseases in later life. Persistent inflammation is probably, the reason why men and women around age of forty start experiencing autoimmune diseases.

1. ANA prevalence among paediatric population

Peter N Mallelson[39], in UK evaluated around thousand paediatric patients and found that autoantibodies were prevalent in childhood populations, with 135 children, living with rheumatological disorder.

2. ANA in reproductive age group women

O Yaddin[40], in his study screened 506 healthy women and 60 women of child-bearing age for antinuclear antibodies. After five year follow-up, 57 of these 60 women were found to have autoantibodies to a variety of autoantigens. Seven of the women had some symptoms that could be associated with the presence of the antibodies, so they pointed the fact that in normal subjects(women aged 22-44 years) high titres of natural autoantibodies are present. They should be followed for a period of atleast 5 years to see if they were at a high risk of developing an overt autoimmune condition.

3. ANA prevalence among elderly population

A study conducted by Juby[41] on 399 elderly patients in comparison with 250 healthy adults by measuring various autoantibodies, suggested that the presence of antibodies directed against nuclear proteins in

elderly patients with chronic illness and connective tissue disorders rather than healthy adults.

3.2.2ANA prevalence in accordance with sex

Among patients with connective tissue disorders, more than three-fourth were females. The reason is unknown, probably because of fluctuating serum hormonal level and predominant immune response by T-helper 2 cells.

Women have a robust immune system as compared to men; and it acts as a double edged sword, protecting them from diseases and attacking them to cause diseases. Females are more prone to urinary tract infections, menstrual periods, pregnancy and suffer from stress, all of which trigger autoimmune diseases. Men, around the age of forty, start to get autoimmune diseases, due to declining testosterone levels[42].

Rus V[43] in Denmark have found fourteen fold higher positivity in females in compared to males.

In Kulalampur, MR Azizah[44] screened serum samples from 93 blood donors. Antibodies to nuclear antigens were assayed by immofluorescence method using human epithelioma cells as substrate. Raised titres were found in 2/19 (10.5%) females and 4/74 (5.40/0) males ($p > 0.05$).

In Brazil, Fernanderz sav *et al*[45]screened samples from 500 blood donors and found that the female donors had high risk of seropositivity for antibodies directed against nuclear antigens.

A study conducted by Hayashi[34] in Japan for disease-specific antibodies directed against nuclear antigens (ANAs) from 2181 residents, inferred that antinuclear antibodies positivity were higher among females.

3.2.3 ANA prevalence among various racial groups

African American, American Indian, or Latino people are more likely than Caucasians to develop autoimmune disorders.

A study done by Cacciplaglia[46], compared migrants to Italy and native Caucasian population. Antibodies directed against nuclear proteins were assessed by fluorescent microscopy. They found that the migrants were having high positive rates for antibodies against nuclear proteins(23.7% vs 8.3%).

3.2.4 Familial predisposition to autoimmunity

Studies have shown that the tendency to develop autoimmune disorders can be inherited.The genes which plays role in the development of autoimmune disorders, can be transmitted vertically and each abnormal genes may give rise to different types of presentations.

Huang C M[47] in Taiwan studied family members and close relatives of different rheumatological disorder and found that there was

higher rates of positivity among 2nd and 3rd degree relatives of patients with Lupus when compared to first degree ones.

Eaton *et al*[48] studied 31 types of connective tissue disorders and found that the disease have higher preponderance in family members.

3.3 ANA prevalence among blood donors, hospital personnel

A study done in Mexico by Marin[49], evaluated three groups of persons- blood donors, persons working in hospital, and relatives of patients with rheumatological disease.

The study population were healthy during the conduct of study. Among 304 individuals screened, 165 were found to have antibodies directed against nuclear antigens.Hospital personnel and relatives of patients with rheumatolgical disorders had higher positive rates, and speckled pattern was the most common finding among them.

Serum of 500 adult blood donors were tested for the presence of autoantibodies by Fernanderz sav et al[45]. Antibodies directed against nuclear proteins were detected in 22.6% of the sera.

3.4 Antinuclear antibodies prevalence among pregnant women

Some of the connective tissue disorders tends to rise during reproductive age groups. During gestational period there will be a exchange of cells between mother and the developing conceptus, which may sustain in their circulation even after delivery[5].This may lead to the development of autoimmune disorder in future .Only few prospective

studies exist to provide accurate information for counselling patients about the risks, outcomes, and appropriate therapies pertinent to pregnancy.

Farnam J[50] screened 214 antenatal mothers with 50 controls to determine ANA positivity.23 antenatal women were found to possess seropositivity to antibodies directed against nuclear antigens while only one patient in control group tested positive. In the antenatal mothers,13.4% of mothers belonged to late gestational period and 9.2% of mothers in mid gestational period. Antibodies directed against deoxyribonucleic acid was present in 2 patients.

Rosenberg[51] evaluated early, mid, and late gestational serum samples from 100 healthy pregnant women, umbilical cord serum samples from each delivery, and for comparison, sera from 76 non-pregnant control subjects were assayed by immunofluorescence by microscopy for the presence of antibody to HEp- 2 cell nuclei at titers of greater than or equal to 1 in 80.

At serum dilutions of 1 in 80, the numbers of samples positive in the early, mid and late gestations and cord sera were 18, 21, 21, and 15, respectively. At serum dilutions of 1 in 160, the numbers of sera positive for antinuclear antibodies in each trimester and in cord sera were 9, 12, 9, and 8, respectively.

Patton[52] in Italy screened samples from 136 women (84 pregnant and 52 nonpregnant) for antibodies directed against nuclear antigens, smooth muscle antigens, by indirect immunofluorescent microscopy. Immunofluorescence assays were considered positive if antibodies were detected at a dilution of serum 1 in 20. The mean ages of the two groups were 32 and 26 years, respectively. Autoantibodies did not differ in the study (46.2) and control groups (40.5).

3.5.1 Antinuclear antibodies prevalence among women with Bad Obstetric History

Garcia *et al*[53] compared the prevalence of antibodies directed against nuclear antigens in the sera from patients with more than 3 miscarriages to that of women with normal gestation. Antibodies directed against nuclear antigens were more prevalent in patients with more than 3 miscarriages(30%) when compared to women with normal gestation (6.6%).

Xu L[54] in Japan compared four groups of women to study ANA prevalence among them. Group A consisted of 30 patients with a history of unexplained fetal losses. Group B consisted of 30 women with "explained" fetal losses (e.g., uterine septum or luteal phase defect). Group C consisted of 61 healthy pregnant women. Group D involved 61 healthy non-pregnant women of reproductive age.

In groups A and B, 40% and 53.3% of the respective patients had antinuclear antibody titers greater than or equal to 1 in 40 dilutions. In groups C and D the frequencies of positive antinuclear antibody titers were 8.2% and 5.6%, respectively.

Nakatsuka *et al*[55] in Japan, studied the efficacy of examining antinuclear antibody (ANA) as a screening test to detect subclinical immune disorders in infertility and sterility. ANA was measured in 116 unexplained infertile or sterile patients.

The ANA positive rate was 43.5% in group A (habitual abortion, n = 23), 38.1% in group B (consecutive miscarriages, n = 21), 30.0% in group C (one miscarriage, n = 10), 16.7% in group D (one or more deliveries n = 12) 22.0% in group E (primary sterility, n = 50), and 22.4% in the control group (n = 54). The positive rate for all the infertile patients (group A+B) was 40.9% and significantly higher than that in for the control group ($p < 0.05$).

Bahar *et al*[56] conducted a controlled study on 103 patients with unexplained recurrent spontaneous abortions for frequency of positive test for anticardiolpin and antinuclear antibodies. The frequency of positive test for antibodies directed against nuclear antigens was 13.6% in persons with recurrent spontaneous abortions compared to 1.2% in control population. No difference was found between first and second trimester aborters in frequency of positive test for either ACA or ANA.

Malinowski[57], in Lodz, compared the frequency of low-level antinuclear antibody titers in 68 women with bad obstetric history (group A) to that in 35 with explained repeated pregnancy losses (group B) and 44 healthy pregnant (group C), and 36 healthy non-pregnant women (group D).

The frequency of antinuclear antibodies positivity in a dilution of 1 in 40 or higher was 51.5% in group A, 34.3% in group B, 6.8% in group C and 5.6% in group D.

A study was done by Remaind[60] in Argentina in 108 females with bad obstetric history against control group of 392 women from general population. They reported 40.7% positivity in women with bad obstetric history and 14.8% positivity in control sera. Antibodies directed against nuclear antigens was the most common association.

Eroglu[61] analyzed the presence of antibodies to phospholipid and nuclear antigens in 72 women with a history of recurrent fetal wastage. An indirect immunofluorescent antinuclear test was performed. Nine women (13.2%) had low levels of antinuclear antibodies, none of which were specific.

In a study conducted in Argentine women[62] with early gestational loss and fertile women, no significant difference in antinuclear antibodies positivity could be attributed to either of the groups.

Ruiz JE[63] in his study, tested Colombian women with poor obstetric performance to antiphospholipid antibodies and antibodies directed against nuclear proteins. Sixty-eight non-pregnant and 25 pregnant women with poor obstetric performance included in the study group was compared with 56 healthy pregnant and non-pregnant females. There was no difference in the seropositivity of antinuclear antibodies in either group.

Bahar *et al*[56] found that less positivity rate for patients with recurrent spontaneous abortions(8%) in comparison to normal multiparous women (10%), in Kuwait population using immunofluorescence by microscopy.

Petri *et al*[64], investigated ANA prevalence in women with three pregnancy loss was around 16% when compared to healthy women it was around 20% using indirect immunofluorescence, from a population of 60 women in each group.

3.5.2 Antinuclear antibodies association among BOH patients in Indian scenario

U.Shankarakumar[7] in Mumbai, studied Fifty patients with sub-fertility or infertility along with 50 healthy antenatal women for various auto-antibodies such as ANA, anti-dsDNA, ANCA,AECA using immunofluorescence and ELISA. 34% of study population tested positive

for autoantibodies.Antinuclear antibodies was present in 12% of patients with infertility compared to 2% among controls.

Usha[13] in Varanasi, studied cohort of 28 female patients with recurrent abortions, which included 8 primary aborters and 20 secondary aborters. Twenty normal fertile female controls were studies for IgG anticardiolipin antibodies by ELISA and ANA by HEp-2 cell nuclei using indirect immunofluorescence methods. Secondary aborters had more increased frequency of anticardiolipin antibody (35%) as compared to primary aborters (12.5%).

3.6 ANA detection by Indirect Immunofluorescence

3.6.1 ANA results by titre-wise distribution

Rosenberg[51] evaluated early, mid and late gestational serum samples from 100 healthy pregnant women, and76 non-pregnant control subjects assayed by indirect immunofluorescence for the presence of antibody to HEp 2 cell nuclei at titers of greater than or equal to 1 in 80 dilutions.

At serum dilutions of 1 in 80, the numbers of samples positive in early, mid, late gestation, and cord sera were 18, 21, 21, and 15, respectively. At serum dilutions of 1 in 160, the numbers of sera positive for antinuclear antibodies in each trimester and in cord sera were 9, 12, 9, and 8, respectively.

Harger [65] in Pennyslavia, USA compared a group of 277 women with recurrent pregnancy loss to that in 299 pregnant controls and 119 non-pregnant controls. The frequency of positive antinuclear antibody tests at a titer of 1 in 40 or higher was 16.3% in cases, 16.6% in pregnant controls, and 16.8% in non-pregnant controls. Increasing the critical titer to 1 in 80, however, led to a statistically significant difference between cases (6.9%) and controls respectively.

A study done in Brazil[45] in 500 blood donors included in the sample, 113 were positive for ANA, prevalence of 22.6% were observed. Among the donors who presented ANA+, 73(64.6%) had a titer of 1 in 40, 23 (20.4%) a titer of 1 in 80, 10 (8.8%) a titer of 1 in 160, and 7 (6.2%) a titer equal or higher than 1 in 320.

Hayashi N[34] investigated 2181 residents in Japan, and antinuclear antibodies positivity rates were 26% and 9.5% at dilutions of 1 in 40 and 1 in 160 respectively.

In Mexican population, Marin[49] studied 304 individuals, of which antibodies directed to nuclear antigens was detected in 165 serum samples (54.3%). The most common titre was 1 in 40 (35.4%), followed by 1 in 80 (13.4%), 1 in 160 (3.2%), and 1 in 320 (1.3%).

3.6.2 Evaluation of ANA detection by various HEp-2 cell pattern

The families of 65 patients with systemic sclerosis were investigated Maddison[66].The patients were subjected to

immunofluorescence technique using human epithelioma substrate. Among 217 individuals who participated in test, 58(27%) had antinuclear antibodies.The most common pattern being speckled(42 persons), followed by nucleolar(13 persons), homogeneous(2 persons) and centromere pattern in one person.

In India, Chopra[67] detected ANA from sera and filter paper blood clots of 21 patients with proven connective tissue disorder using indirect immuno-enzyme method.The most frequent ANA patterns was homogenous, speckled, and centromere.

I E Afmann[68] in South Africa, conducted a study which consisted of 46 patients who tested positive for antibodies against nuclear antigens. Among 46 women, 28 had an antinuclear pattern, 13 an anti-cytoplasmic pattern and 5 anti-nucleolar.

Total of 304 individuals, were screened by Marin[49], fluorescence was detected in 165 serum samples (54.3%). The most frequent pattern was speckled (50.3%).

3.7 Comparison of ANA detection by IFAT and ELISA

In Japan Kumagai[69] tried to differentiate 258 connective tissue disease (CTD) patients (except rheumatoid arthritis) from 257 healthy subjects between Enzyme Linked Immunosorbent assay and indirect immunofluorescence by Microscopy. The true positives and true negatives of Enzyme Linked Immunosorbent assay were 84% and 94%,

respectively, while those of IF-ANA at a titre of 1 in 160 were 81% and 87%. The receiver operating characteristic (ROC) analysis concluded that Enzyme Linked Immunosorbent assay was better than IF-ANA.

Divate *et al*[70] have studied samples from 96 patients with connective tissue disorders, by Enzyme Linked Immunosorbent assay of which 53 samples were positive and 43 were negative for immunofluorescence. The sensitivity, specificity, predictive values for positive(PPV) and negatives(NPV) of ELISA were 90.7%, 85.7%, 89.1%, 87.8% respectively.

In Spain, Gonzalez[71], investigated serum samples from 74 healthy individuals, 119 patients with defined systemic autoimmune diseases, 26 patients with other autoimmune diseases, and to which 490 routine samples were also analysed by ELISA.

For systemic lupus erythematosus (SLE) patients, the COBAS-ANA(ELISA kit) best efficiency was obtained with a cut-off of 0.9, with a sensitivity of 97% and a specificity of 88%, whereas the best IFA-ANA efficiency was obtained with 1 in 80 dilution, giving a sensitivity of 90% and a specificity of 99%. There were no differences between areas under receiver operating characteristic curves for two methods. For other systemic and non-systemic autoimmune diseases sensitivity and specificity of COBAS-ANA were similar or higher than that of 1 in 160 IFA-ANA titer.

Tonuttia *et al*[72] in Italy, studied the efficacy of five types of ELISA kits in comparison with imunofluorescence to identify antibodies directed against nuclear proteins. The seropositivity of immunofluorescence technique was 92%, whereas five ELISA kits showed positive results which ranged from, 74 to 94%. ELISA kits were able to identify all antibodies which produces homogeneous and speckled pattern, in contrary to nucleolar pattern.

Paz[73] in Israel, compared Enzyme Linked Immunosorbent Assay with IFAT method, from eighty five patients with SLE and 51 healthy volunteers. The samples were tested by the above two methods at 1 in 40 and 1 in 160 dilutions.Those who were positive for indirect immunofluorescence showed better reactivity for Enzyme Immunoassay compared to those who had negative results.

Enzyme Immunoassay was compared to indirect immunofluorescence by Op De Beecke[74](2011) to identify antibodies directed against nuclear proteins in various rheumatological disorders.The sensitivity of EliA CTD Screen for systemic lupus erythematosus, systemic sclerosis, primary Sjögren's syndrome, mixed connective tissue disease, and inflammatory myopathy was 74%, 72%, 89%, 100%, and 39%, respectively.

Dipti[75] in Bangladesh evaluated immunofluorescence method and enzyme immunoassay in 40 patients, of which 20 had Lupus since younger age, and 20 patients had other autoimmune disorders excluding Lupus.The antinuclear antibodies positivity rate was 100% by IFAT and 55% by enzyme immunoassay in patients with Lupus since younger age.Immunofluorescence have more diagnostic capacity than enzyme immunoassay in antinuclear antibodies positivity.

3.8 Techniques used for detection of specific ANA

- **Crithidia luciliae assay**

Thirty four systemic lupus erythematosus patients were screened by Sommerfield[76] using radio-labelled assay, passive haemagglutination, and Crithidia Luciliae assay.Among these methods, results of Farr assay and Crithidia luciliae assay were similar, while the findings in haemagglutination method were quite dissimilar from the other two methods.

- **Indirect Immunoenzyme method**

Chopra A[67] compared simple Indirect immunoenzyme with the help of light microscope with indirect immunofluorescence. In his study, he identified antinuclear antibodies from serum samples and filter paper blood clots(FPBC) of patients who were diagnosed with proven rheumatolgical disorder using IIE.Both FPBC and serum samples were collected from 10 patients.The results of two methods was similar.ANA

patterns(homogenous, speckled, centromere) from both FPBC elutes and serum are comparable.

❖ **Passive haemagglutination**

The results of passive haemagglutination were evaluated by Niemhom[77], with those obtained by immunofluorescence in 169 patients with active SLE and inactive SLE.59 sera were positive and 91 sera were negative by both methods. Five sera were negative by haemagglutination but positive by immunofluorescence. Fourteen sera with low haemagglutination titer were negative by immunofluorescence.

The correlation between the results obtained by both methods were highly significance with contingency coefficient of 0.61 and correlation coefficient between the results of 78 sera positive by both or either method was 0.74.

❖ **Line blot assay**

Damoiseaux[29] compared line immunoassay with counterimmuno electrophoresis (in house CIE) and enzyme immunoassay for the presence of autoantibodies.148 patients with proven rheumatological disorders and 40 controls participated in the study.The true positive rate for line immunoassay was(17.9%) , for enzyme immunoassay (11.4%) and for inhouse CIE (8.3%).

Serum samples of patients from a random South Indian population[1] who sought medical help for rheumatic disease were subjected for ANA

testing by indirect immunofluorescence (IIF) method and line immunoassay during the study period of 27 months. Serum samples were initially processed for indirect immunofluorescence, and further for line immunoassay.

The antinuclear antibody indirect immunofluorescence (ANA - IIF) patterns obtained were projectable to visualize a certain spectrum of specific antibodies such as homogenous (45.5%) with dsDNA, nucleosomes, histones, SSA / Ro-52, RIB and RNP / Sm, speckled pattern (35.6%) with Sm, RNP, SSA/Ro-52, SSB, Sm and RIB; nucleolar pattern with Scl-70, Sm, RNP and centromere pattern with CENP-B.

❖ **Multiplex Immunoassay(MIA)**

Assay specificity was evaluated by testing samples from 50 blood donors and 30 specimens from samples with patients having cryoglobulinemia hypergammaglobulinemia, or IgG and IgM monoclonal immunoglobulins, complement-associated diseases. To evaluate assay sensitivity,142 samples from patients with systemic rheumatic disease that were tested previously by routine methods. The sensitivity and specificity were 99.1% and 100%[27,28].

Hayashi *et al*[34] compared Multiplex Immunoassay and immunofluorescence (IF) assay in 492 persons from community and 307 persons with autoimmune disease. The sensitivity and specificity of the IF method at a titre of 1 in 40 were 92% and 65%, respectively, vs 93%

and 79% for the MIA at a cut-off of 0.6, but at higher titres in IFAT and higher cut-off with MIA, the results are similar to some extent.

❖ **Antigen Microarray**

Microarray technology allows the simultaneous analysis of thousands of molecular parameters. Microarrays are made using either on-chip synthesis strategies or with an arrayer based on contact-printing or ink-jet technology[4,28].The recognition of autoantibodies may be achieved using fluorescence or radio-active labelling, chemiluminescence, mass spectrometry or electrochemical methods.

3.9 Various treatment options for women with RPL

1. Aspirin/heparin therapy

Acetyl salicyclic acid and low dose heparin are first line drugs for women with RPL[5].Aspirin is more preferred because it crosses placenta.

Reznikoff- Etievant[78] investigated 678 healthy patients with recurrent gestational loss of which 161 autoantibody-negative and 53 autoantibody -positive women received prednisone and aspirin and 63 autoantibody-negative women received aspirin alone. In autoantibody-positive patients treated with prednisone and aspirin the success rate was 84.9% .

2.Prednisone

The mechanism action of steroids is that they suppress the inflammatory process and stabilizes the cell. Out et al[79](1992) found 36% pregnancy loss rate in patients treated with prednisone compared with 22% pregnancy loss in patients treated with both prednisone and anti-coagulation therapy.

3. Intravenous immunoglobulin (IVIg) therapy

The mechanism by which gammaglobulin acts is that it alters T-cell subsets,modulates cell mediated immune response,neutralises cytotoxic effect of maternal immune response against fetus.It could be safely used in persons with side-effects to aspirin and heparin[5].

A study done by Stricker[53] to evaluate the usefulness of gammaglobulin in 47 women with recurrent fetal wastage, found 75% successful pregnancy rate.

Christiansen O B[80] in his study reported that patients who were given intravenous immunoglobulin treatment for sub-fertility, had better success rate compared with placebo.

4. Lymphocyte immunotherapy

Lymphocyte immunotherapy helps to decrease the level of maternal interleukin receptors, shift to Th-2 type immunity and causes suppression of natural killer cell activity[5].

5.1a, 25 –dihydroxy-vitamin-D3 (VD3] therapy

Vitamin D3 acts as a immunomodulatory agent.The mechanism of action is not known, probably it is thought to down-regulate the production of T-helper cytokines.

Thus it is known that, changes in immune system occur during antenatal period.The presence of antinuclear antibodies in women is either an expression of subtle immune disorder or is an immunologic epiphenemenon is not clearly defined.On reviewing the literature, it is suggested that ANA detection would be of some value in identifying patients with these disorders, as the condition is treatable.

Materials and Methods

4. MATERIALS & METHODS

4.1 Materials

4.1.1 Study period

The present study was conducted from the period of August 2011 to September 2012. The study population comprises mainly of pregnant women from 19-40 years, attending Antenatal clinic and inpatients in Obstetrics and Gynaecology Department, Tirunelveli Medical College Hospital.

4.1.2 Sample size

This is a case-control study comprising of equal number of cases and controls, each comprising 60 women of reproductive age group.

4.1.3 Study group

The study population comprises of 60 pregnant women from age group 19-40 years

- With history of 2 or more spontaneous abortions.
- It also includes women with past history of still-births, intra-uterine death or early neonatal deaths.

  The population was carefully selected that women in study group has no live issues.

4.1.4 Control group

The control group comprises of 60 multiparous pregnant women from age group 19-40 years, with no history of abortions.

4.1.5 Exclusion criteria

This group was carefully selected to exclude women:

- history of heart disease, anaemia,renal failure
- history of Rh-incompatability
- history of sexually transmitted diseases, or TORCH infections
- history of uterine fibroids, endometriosis, PID, uterine anomalies- septate uterus, bicornuate uterus.

4.1.6 Ethical clearance

Since the study involves blood samples from pregnant women, institutional ethical clearance was obtained.

4.1.7 Informed Consent

Informed written consent was obtained from all pregnant women involved in the study, both study and control group.

4.1.8 Questionnaire

Questionnaire was given to all persons which included details about, their name, age, spouse age, last menstrual period, any complaints like-abdominal pain, draining per vaginam, bleeding per vaginam, history of systemic illness, menstrual history- age at menarche, duration of

menstrual cycle, obstetric history- which includes their gravida, abortions- spontaneous or induced, nature of past delivery- full term/ pre-term, normal vaginal delivery or Caessarean section, any contraceptive usage, sexually transmitted infections any, drug intake for some disease.

4.1.9 Sample collection

Under strict aseptic precautions,3-5ml blood was collected from both study group and control group, using sterile syringe.

4.1.10 Separation of serum from sample

Blood was collected in sterile glass tubes, and sample container was kept in slanting position at room temperature for 30 minutes. From the specimen vial, serum was transferred to sterile screw capped leak proof tube.

Lipaemic, lysed, deteriorated samples were discarded.

4.1.11 Storage of serum

Serum vials were packed in a second tightly capped unbreakable container, surrounded by adequate packing material and stored at -20˚C deep freezer.

4.1.12 Biomedical waste management

As the materials handled were highly infectious, proper biomedical waste management was followed throughout the study.

4.2 Indirect Immunofluorescence Hep-2 cells (ANA-Hep-2)

Antibodies directed against nuclear antigens bind to the corrsponding antigen's present in human epithelioma cell type-2 substrate.Over this antigen-antibody complexes, fluorescein tagged anti-human IgG is added,which is visualised by fluorescent microscope.

4.2.1Principle of the method

4.2.2 Composition

A. Slides: Hep-2 cells cultured on each well.

B. PBS(10X): Sodium phosphate 112.5mmol/L, potassium phosphate 30mmol/L, sodium chloride 1.15mol/L, sodium azide 0.95g/L, pH 7.2

C+. ANA-Hep-2 positive Control: Human serum containing anti-nuclear antibodies(ANA) homogeneous pattern, sodium azide 0.95g/L

C-. Negative Control: human serum, sodium azide 0.95g/L

D. IgG FITC/Evans: Goat anti-human IgG conjugated with fluorescein isothiocyanate(FITC), Evans blue 0.01g/L, sodium azide 0.95g/L

E. Mounting Medium: Mowiol 12%, Glycerol 30%, Tris 20mmol/L, sodium azide 0.95g/L

F. Blotting paper

4.2.3 Storage

Store at 2-8˚C.

4.2.4 Reagent preparation

PBS: Reagent was diluted 1 in 10 with distilled water.

All other reagents were provided ready to use.

4.2.5 Additional equipment

- Moist chamber
- Wash tray
- Coverslips 24*60mm
- Fluorescence microscope equipped with a 495nm excitation filter and a 525nm emission filter for FITC visualisation.

4.2.6 Samples

Samples were diluted one in eighty in phosphate buffer saline before the assay.

For titration of positive samples, two-fold serial dilutions starting 1/160 in PBS were made.

4.2.7 Procedure

1. Reagents and samples were allowed to come to room temperature.
2. A drop(25µL) of diluted sample or control was placed on each slide(A) well, making sure that it was completely covered(note 1).
3. The slide were incubated for 30 minutes at room temperature(15-30˚C) into a moist chamber.

4. Sample drops were drained off by gently tapping the inclined slide. Cross-contamination of the sera were avoided.
5. The slide was gently rinsed with PBS.
6. The slide was thoroughly washed by immersing in washing tray filled with PBS for 5minutes. PBS is changed and wash was repeated.
7. The slides were carefully dried off by using the blotting paper provided. The substrate was kept moist along the procedure.
8. One drop of reagent D was placed on each well. The slide was incubated for 30 minutes at room temperature(15-30˚C) into a moist chamber.
9. Wash (step 6) and dry (step 7).
10. Several drops of reagent E was placed on the slide and covered with a cover-slip avoiding the formation of air-bubbles.

4.2.8 Reading

The slide were examined using low power and high power of fluorescent microscope.

The slides were read as soon as procedure is over.

The area, between the centre and edge were selected for visualising.

Uniform spacing between cells and nuclei give a better picture.

4.2.9 Quality control

Positive control and negative control provided with the kit should be tested together with the patient samples, in order to verify the assay performance.

4.2.10 Assay characteristics

IgG FITC/Evans and IgG FITC conjugates are valued against the WHO International Standard for FITC labelled sheep anti-human immunoglobulin.

4.3 ELISA kit

4.3.1 Principle of test

Specific anti-nuclear antibodies(ANA) in the sample bind to the antigens immobilized on the microwell surface. In a second incubation, a conjugate of horseradish peroxidase-labeled immunoglobulins to human IgG binds to surface-bound antibodies. Finally, 3,3',5,5'-tetramethylbenzidine(TMB) with hydrogen-peroxidase was added to each well as enzyme substrate and, after color development, the enzymatic reaction was stopped with hydrochloric acid. The yellow product formed was measured in terms of absorbance units at 450nm, and it is proportional to the amount of antibodies present in sample.

4.3.2 Contents and Composition

A) Concentrated Washing Buffer,50ml

Concentrated phosphate saline buffer, sodium azide15mmol/L

B) Sample diluent 100ml

Tris buffer, sodium azide 15mmol'L

C)Positive Control 1.5ml

Provided ready to use. Serum with ANA, sodium azide 15mmol/L

C)Negative Control 1.5ml

Provided ready to use. Serum free of ANA, sodium azide 15mmol/L

CO. Cut-Off Standard 1.5ml

Provided ready to use .Serum with ANA, sodium azide 15mmol/L

D)Conjugate.15ml

Horseradish peroxidase-labeled polyclonal rabbit immunoglobulins to human IgG

E) Substrate.15ml

3,3'5,5'-tetramethylbenzidine(TMB)

F) Stop solution.15ml

Hydrochloric acid 1.0mol/L

G) Microplate

12modules of 8 wells each coated with a mixture of purified SSA 52, SSA 60, SSB(La), Sm/RNP, RNP-70, RNP-A, RNP-C,SmBB', SmD, SmE, SmF, SmG, Scl70,Jo1, dsDNA,ssDNA, polynucleosomes,

mononucleosomes, histone H1, histone H2A, histone H2B, histone H3, histone H4, PMScl-100, and centromere B.

4.3.3. Storage

Store at 2-8˚C.

4.3.4 Reagents and Preparation

Washing buffer

Concentrated washing Buffer was diluted with distilled water in the proportion 1/20and mixed throughly. About 50ml of washing buffer were used per strip.

All other reagents were provided ready to use

4.3.5 Additional Equipment

- Moist chamber
- Multi-tip aspirator or automatic washing equipment for microplates
- Microplate reader or photometer with microcuvette, with a 450+/-10nm filter.

4.3.6 Samples

Serum or plasma were collected by standard procedures. Sample was diluted with 1/100 with sample diluent. Fresh sample dilutions has to be used always.

4.3.7 Procedure

1. All the reagents and microwells were allowed to warm up to room temperature.
2. The microplate package(M) was opened and the required amount of wells were taken out.
3. 100µL of each diluted sample, Cut-off standard(CO), Positive Control(C+) and Negative Control (C-) were pipetted into different wells.
4. Wells were placed in a moist chamber and incubated for 30 minutes at room temperature.
5. The contents were aspirated and the well's were washed 3 times with 300µL of washing buffer for atleast 10 seconds.
6. 100µL of Conjugate(D) was pipetted into all wells.
7. The wells were placed in the moist chamber and incubated for 15mins at room temperature.
8. The wells were washed as in step 5.
9. 100µL of Substrate(E) was pipetted into all wells.
10. The wells were placed in moist chamber and incubated for 15 minutes at room temperature.
11. 100 µL of stop solution was pipetted into all wells and incubated for 5minutes at room temperature.

12. The absorbance of the contents of each well were read at 450nm.The colour is stable for atleast 30minutes.

4.3.8 Calculations

The absorbance ratio was calculated as follows:

$$\text{Absorbance ratio} = \frac{A_{450nm}\ \text{sample}}{A_{450nm}\ \text{cut-off}}$$

When the absorbance values obtained are over the upper limit of the microplate reader range, samples should be further diluted with Sample diluent and reassayed.

4.3.9 Reference values

Samples with absorbance ratio higher than 1.2 are to be considered positive.

Samples with absorbance ratio lower than 1.0 are to be considered negative.

Samples with absorbance ratio between 1.0 and 1.2 would be considered as border line and repeat testing is recommended.

These values were given for orientation only. It is recommended that each laboratory establishes its own reference values.

4.3.10 Quality control

- ❖ Absorbance of positive control should be higher than 0.600.
- ❖ Absorbance of negative control should be lower than 0.300.
- ❖ Cut-Off Standard should be higher than 0.300

4.3.11 Interferences

Hemoglobin (1000mg/dl), bilirubin (40mg/dl) and triglyceride (3000mg/dl) do not interfer. Other substances and drugs may interfere.

Results

5. RESULTS

5.1 Study group

A total of 120 pregnant women were included in the study.60 pregnant women with bad obstetric history were evaluated against 60 mutiparous women. This study was conducted for a study period of one year from August 2011 to September 2012. The study population mainly consisted of women attending antenatal clinic and inpatients in Obstetrics and Gynaecology Department, Tirunelveli Medical College Hospital, Tirunelveli.

5.2 Statistical Analysis

Data regarding the cases and controls were described in terms of percentages. Sensitivity, specificity, positive predictive value, negative predictive value of ELISA was done. The cases and controls were statistically analysed for significance by Chi-squared test. The Chi-squared test was calculated for the analytic assessment by SPSS 20 version software. The differences were considered to be statistically significant when the p value obtained was less than 0.05.

5.3 Result Analysis

Table-1

Age-wise distribution of BOH cases and controls

Age(in yrs)	No of cases	Percentage of cases	No of controls	Percentage of controls
16-20	7	11.6	5	8.33
21-25	13	21.66	14	23.33
26-30	21	35	23	38.33
31-35	12	20	11	18.33
36-40	7	11.6	7	11.6
Total	60	100	60	100

Table 1 shows the age distribution among cases and controls in the present study which shows that, women between the age group 26-30 comprises maximum number which includes 21 cases and 23 controls respectively.(fig 1).

Fig – 1

Age-wise distribution of cases and controls

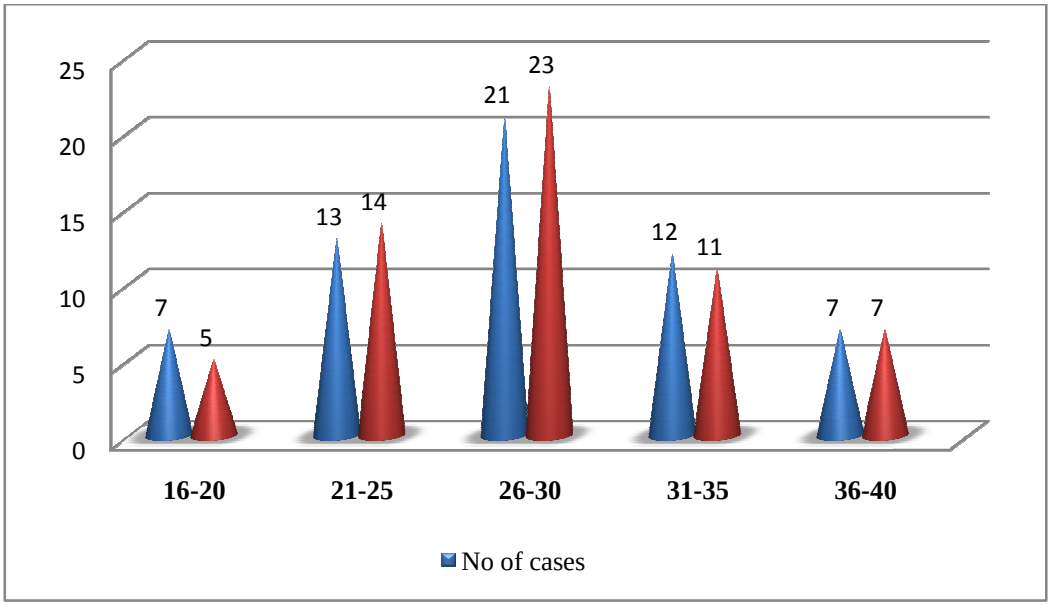


Table - 2

ANA positivity among cases and controls

Samples	Total number	ANA positivity	
		number	Percentage
Cases	60	18	30
Controls	60	5	8.3

Table 2 shows distribution of ANA positivity among cases and controls. Among 60 women with BOH, 18 (30%) were found to be positive. Among controls 5(8.3%) women were positive.(fig 2)

Fig – 2

ANA positivity among cases and controls

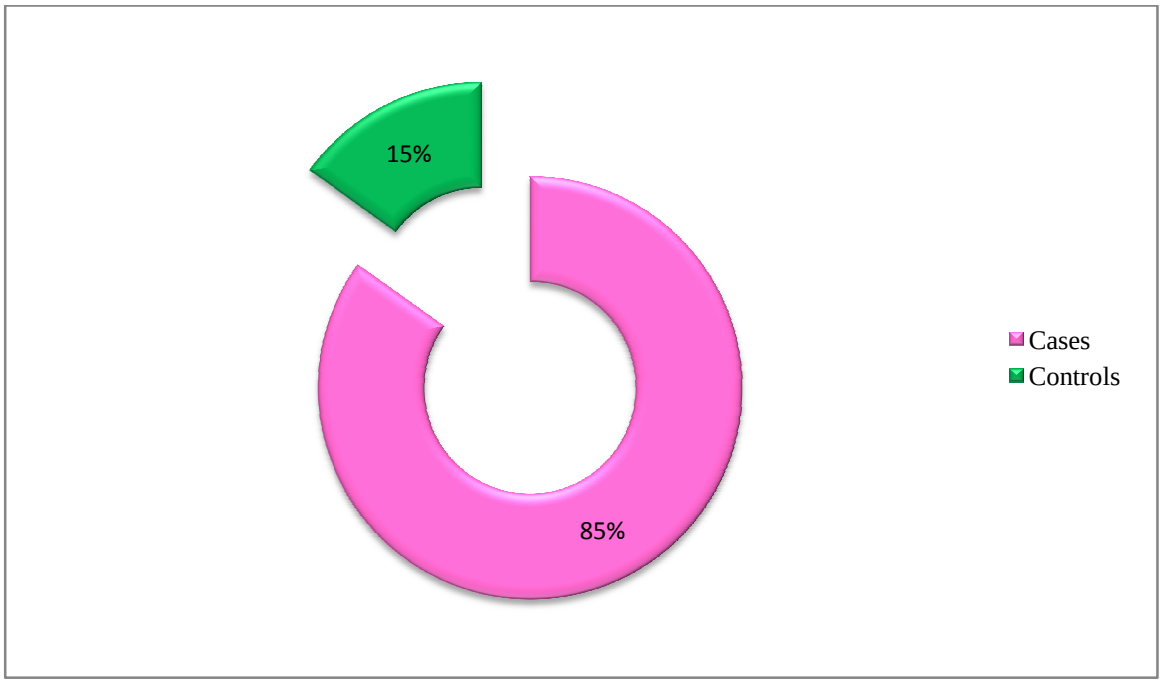


Table - 3

Age-wise distribution of BOH women with ANA positivity

S.no	Age in yrs	No of samples tested	ANA positive cases		ANA negative cases	
			Number	Percentage	Number	Percentage
1	16-20	7	1	5.55	6	14.28
2	21-25	13	3	16.66	10	23.80
3	26-30	21	8	44.44	13	30.95
4	31-35	12	6	33.55	6	14.28
5	36-40	7	-	-	7	16.69
	Total	60	18	100	42	100

This table shows age-wise distribution of BOH cases and anti-nuclear antibodies positive among them. Of the 7 samples tested from age-group 16-20yrs, 1sample is positive and remaining were negative. Of the 13 cases tested 3 were positive and 10 were negative in the age-group 21-25 yrs. From 26-30yrs, 21 samples were tested, of which 8 samples were positive and remaining 13 tested negative. Twelve individuals were tested from age-group 31-35 yrs,6 were positive and remaining 6 were negative. Among 7 cases tested from age-group 36-40 yrs, none were positive.(fig 3)

Of the total 18 cases, which were positive 8 (44.44%) women were in the age group 26-30, 6(33.5%) women were in the age group 31-35,3

(16.66%) women were in the age group 21-25, and 1(5.55%) was in the age group 16-20 yrs, while in women with 36-40 yrs, none showed positivity

Of the 42 negative women with history of BOH, 6 (14.28%), 10 (23.8%), 13 (30.95%), 6 (14.28%),7 (16.69%) were in the age-group 16-20yrs, 21-25yrs, 26-30yrs,31-35yrs, 36-40 yrs respectively. The mean age among cases is 28 years.

Fig - 3

Age-wise distribution of BOH women with ANA positivity

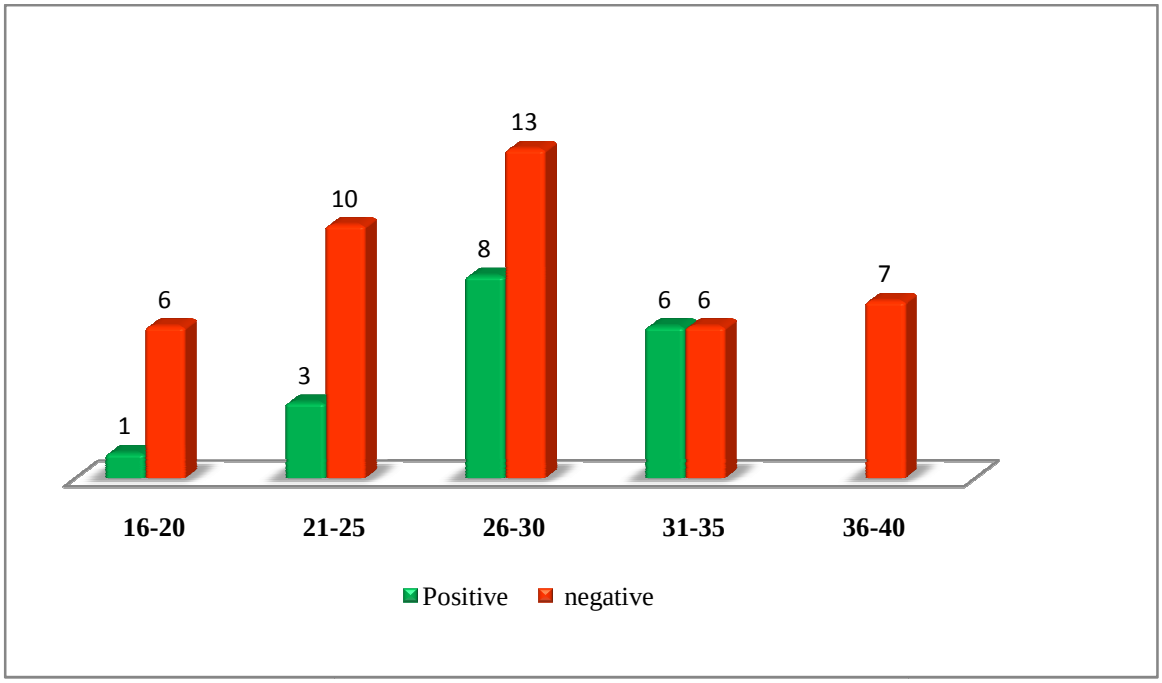


Table - 4

ANA positivity based on obstetric code

Gravida	No of samples tested	No of ANA positives
G3 (n=42)	42	8 (44.44%)
G4 (n=14)	14	7 (38.88%)
G5 (n=2)	2	2 (11.11%)
G6 (n=2)	2	1 (5.55%)

This table shows seropositivity of women with BOH based on their obstetric code. Of the total 42 women with 3rd gravida, 8 (44.4%),women were ANA positive.14 women with 4th gravida, 7(38.88%) were ANA positive. Among 5th and 6th gravida women, with each comprising two, 2(11.11%) and 1(5.55%) were positive respectively.(fig 4).

Fig - 4

ANA positivity based on obstetric code

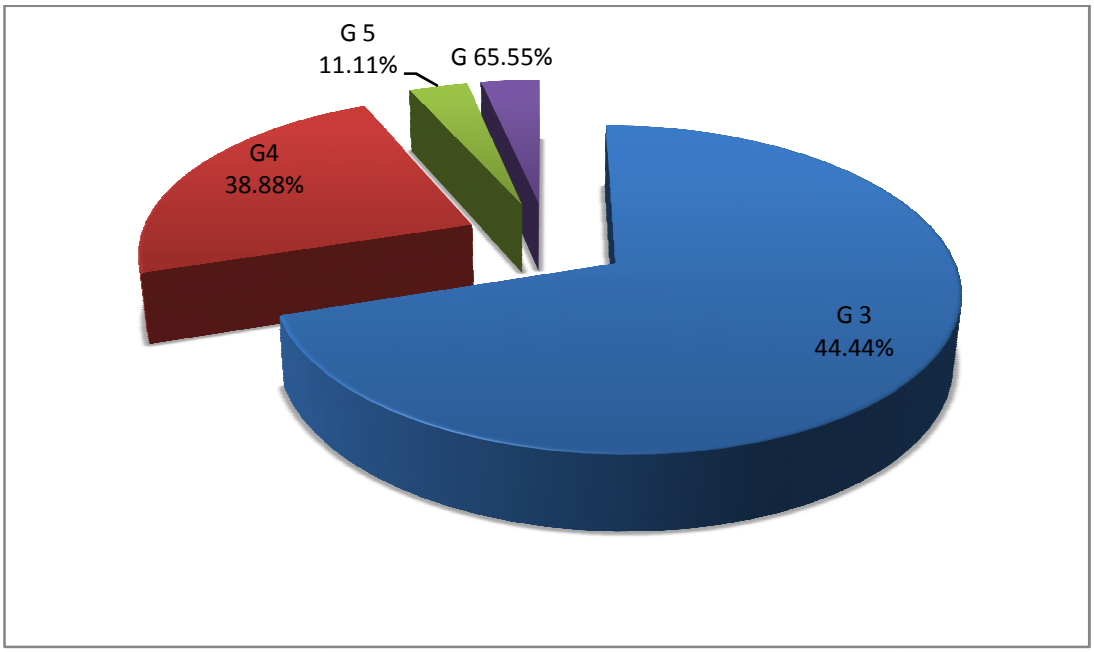


Table - 5

Seropositivity of ANA based on number of abortions

No of abortions	ANA positive	Percentage ANA positive	ANA negative	Percetage ANA negative
≥2 (n=46)	11	23.9	35	76.1
<2 (n=14)	7	50	7	50

P value > 0.05 (Not significant)

The above table shows correlation of ANA positivity with number of abortions. Among women with less than 2 abortions, 11(23.9%) were ANA positive and 35(76.1%) were ANA negative. Among women with more than 2 abortions, 7 (50%) were ANA positive and 7 (50%) were ANA negative . The association between ANA positivity and number of abortions is not statistically significant.(P>0.05).(fig 5).

Fig - 5

Seropositivity based on number of abortions

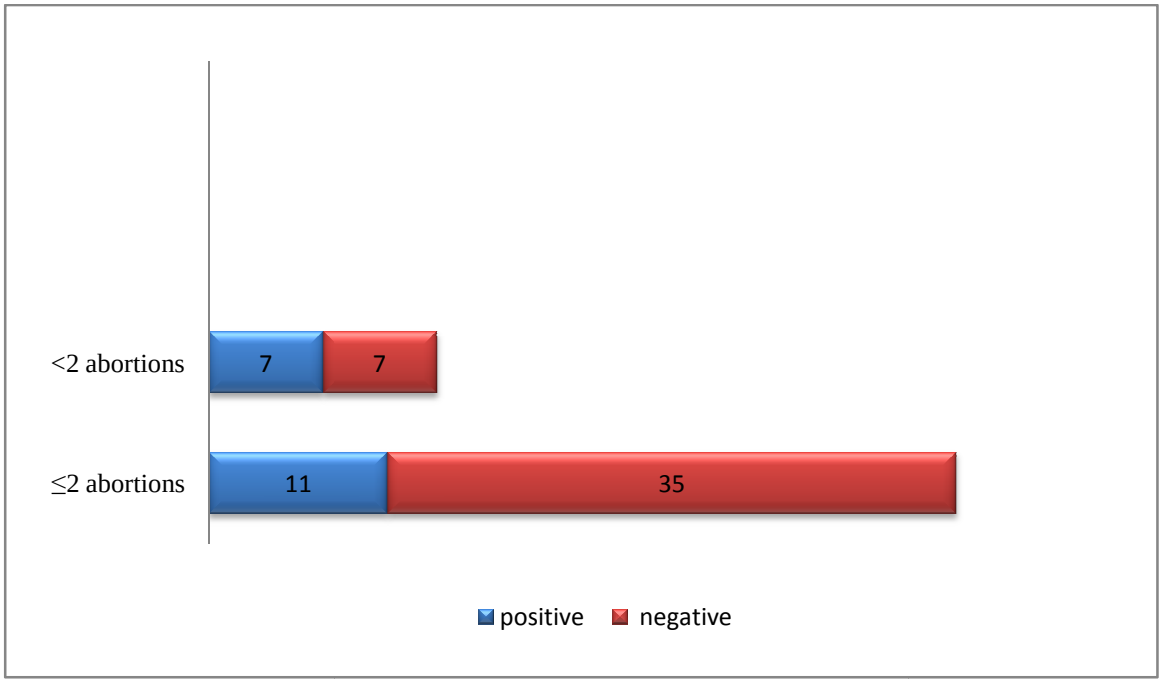


Fig – 6

Distribution of cases based on trimester of pregnancy

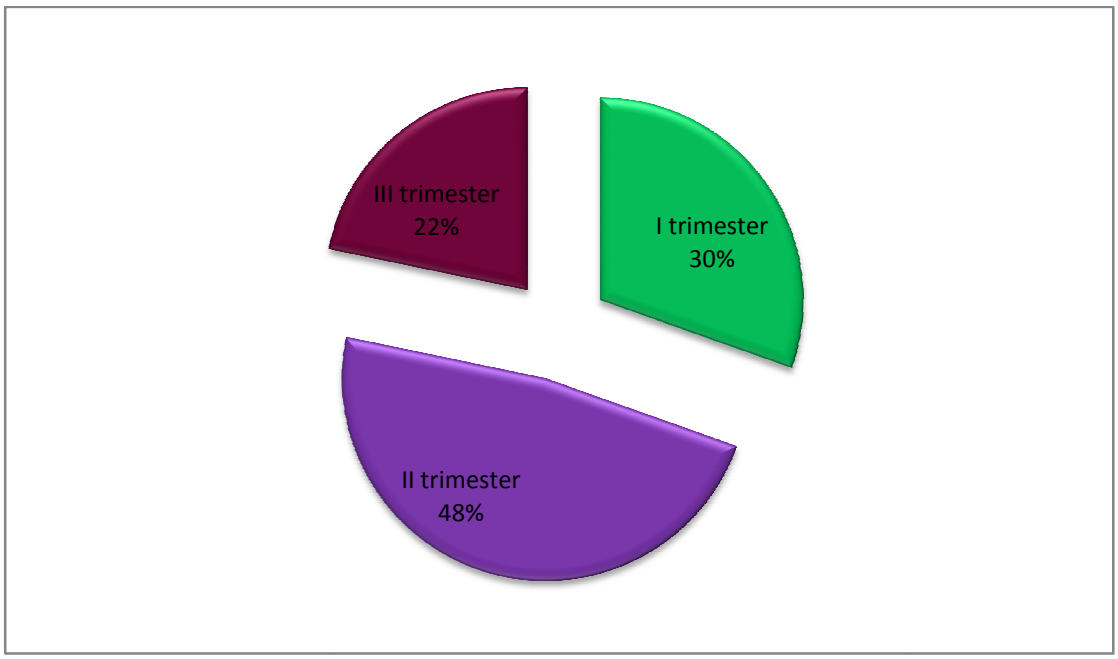


Table -6

Correlation of ANA based on trimester of pregnancy

Trimester of pregnancy	ANA positive	Percentage ANA positive	ANA negative	Percentage ANA negative
1st trimester (n=15)	6	40	9	60
2nd trimester (n=37)	11	29.7	26	70.3

P value >0.05 (not significant)

The above table shows evaluation of ANA positives based on trimester of pregnancy. Of the 15 women in 1st trimester, 6 (40%)were ANA positive and 9 (60%) were negative. Of the 37 women in 2nd trimester, 11 (29.7%) and 26 (70.3%) were ANA positive and negative respectively. The association between ANA positivity and trimester of pregnancy shows no statistical significance.(P>0.05).(fig 6,7).

Fig – 7

Correlation of ANA positivity based on trimester of pregnancy

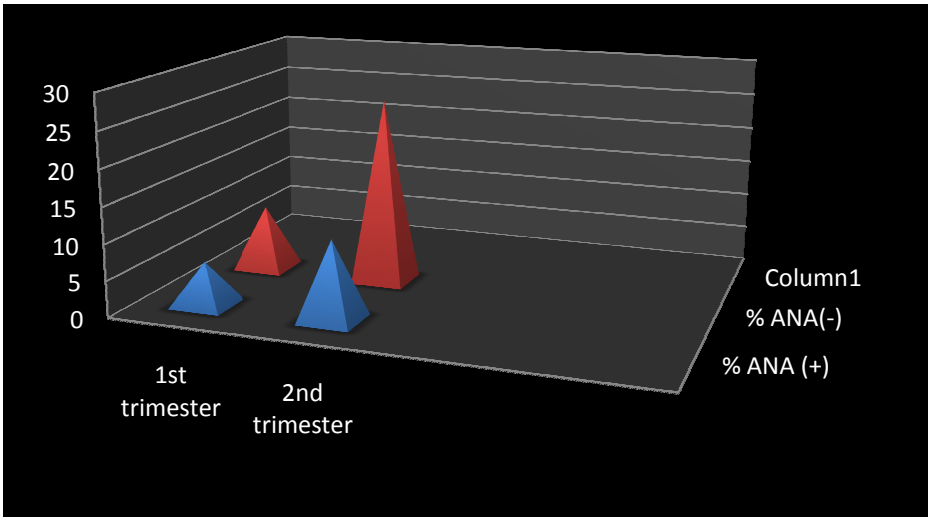


Fig – 8

Co-morbid conditions in women with BOH

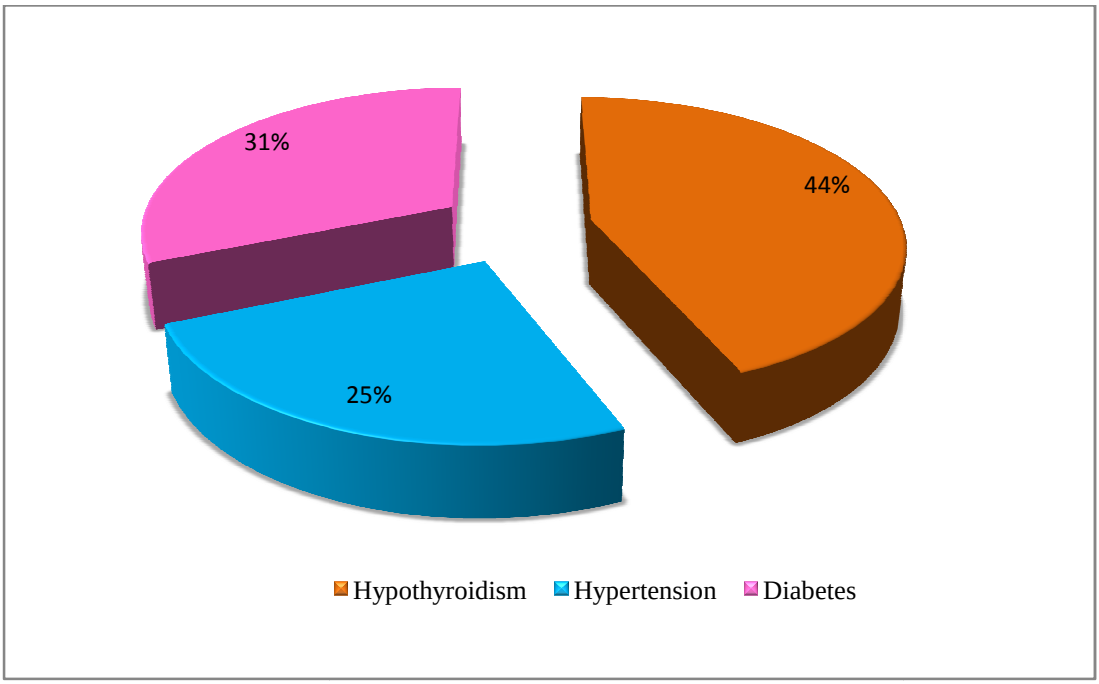


Table 7.

Association of BOH cases with co-morbid conditions

Co-morbid disesases (hypothyroisism, HT, GDM)	ANA positive	Percentage positivity	ANA negative	Percentage positivity
Present (n=16)	10	62.5	6	18.18
Absent (n=44)	8	37.5	36	81.82

P value <0.001 (Statistically significant)

The above table shows association of other systemic disease which includes hypothyroidism, hypertension, diabetes mellitus with the ANA results in present pregnancy. Among 16 cases, with other systemic

disease, 10 were ANA positive and 6 were ANA negative.Eight patients who showed ANA positivity, did not have any associated disease. The association between persons with co-morbid conditions and their ANA results were considered to be extremely statistically significant.(P<0.0001) (fig 8,9)

Fig – 9

Association of BOH cases with co-morbid conditions

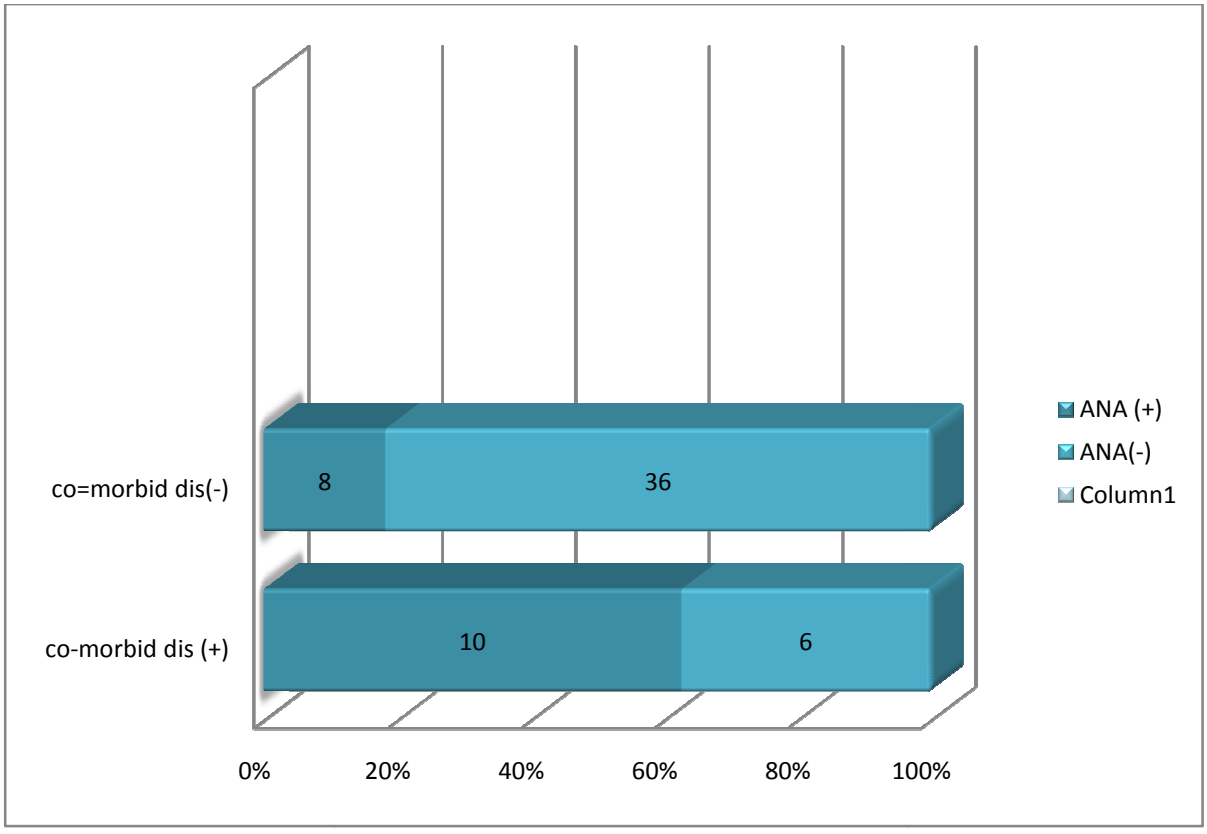


Table - 8

Evaluation of ANA among cases and controls using Immunofluorescence

	IFAT (+)		IFAT(-)	
	Cases	**Percentage**	**Cases**	**Percentage**
Cases (n=60)	18	30	42	70
Controls (n=60)	5	8.3	55	91.66
Total	23	38.3	97	

P value < 0.001(Statistically significant)

Among 60 cases with BOH, 18(30%) tested positive for ANA by immunofluorescence and 42(70%) were ANA negative. Of the total 60 multigravid women, 5(8.3%) of them tested positive for ANA by IFAT and the remaining 55(91.66%) were negative. The association between women with BOH and multigravid controls,using immunofluorescence was considered to be statistically significant.(fig 10)

Fig – 10

Evaluation of ANA among cases and controls using Immunofluorescence

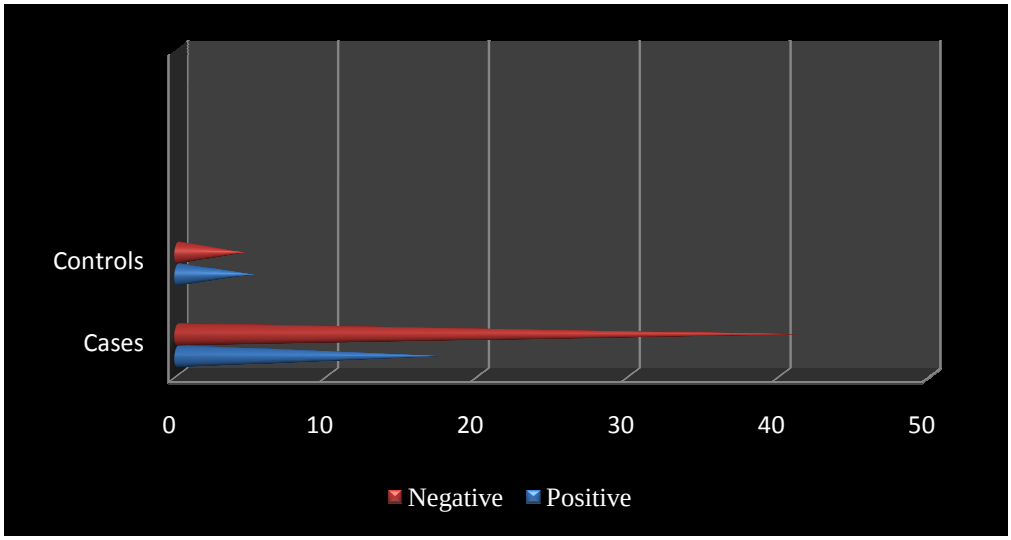


Table - 9

Distribution of HEp-2 cell pattern among IFAT positives

HEp-2 cell pattern	No of positives	Percentage positivity
Homogenous	8	44.44
Anti-centromere	6	26.08
Fine speckled	4	17.39
Coarse speckled	3	13.04
Others- Vimentin, Nuclear rim	2	8.69

This table shows distribution of HEp-2 cell pattern among the 23 IFAT positives which includes both cases and controls.Of the 18 positives, homogenous pattern were seen in 8(44.44%), anticentromere pattern in 6(26.08%), fine speckled in 4(17.39%), coarse speckled pattern in3(13.04%), vimentin and nuclear rim pattern were seen in 2(8.69%) patients respectively.(fig11)

Fig - 11

Distribution of HEp-2 cell pattern in BOH cases

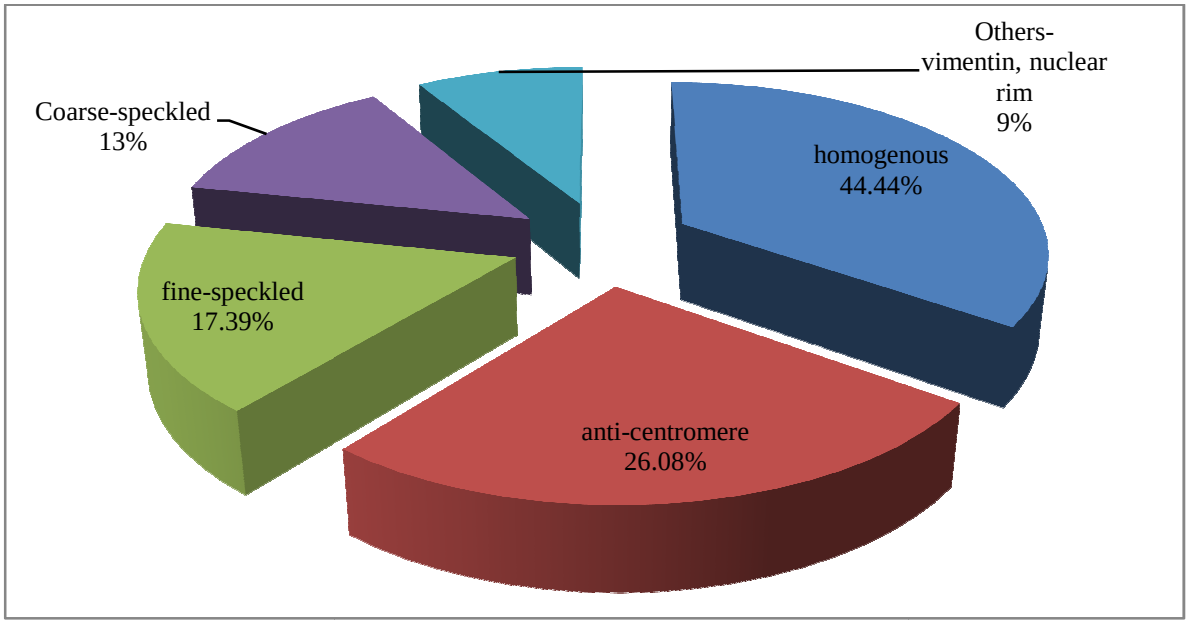


Table - 10

Grading of fluorescence intensity among IFAT positives

Grading	No of positives	Percentage positivity
3(+)	5	21.7
2(+)	6	26
1(+)	11	47.8
Borderline	1	4.34

This table shows grading of fluorescence intensity among the 23 positive patients. 3(+) positive were seen in 5(21.7%) patients, 2(+) positive were seen in 6(26%) patients,1(+) positive in 11 (47.8%) patients and borderline positive in 1(4.34%) patient.(fig 12)

Fig – 12

Grading of fluorescence intensity among IFAT positives

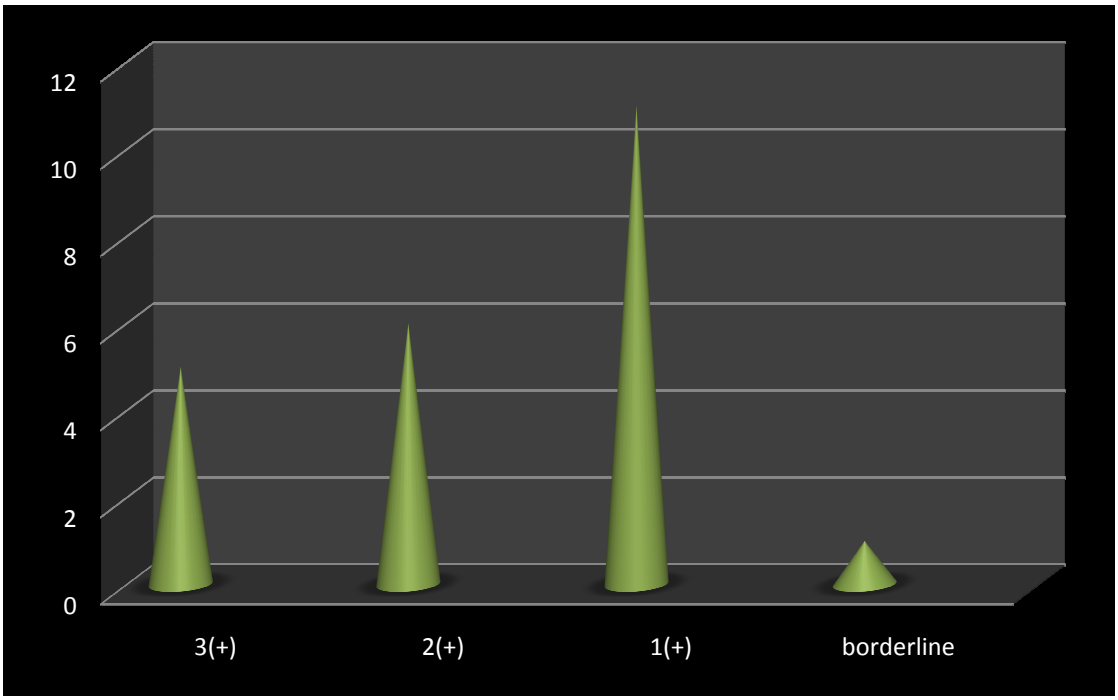


Table - 11

Evaluation of seropositivity among BOH cases and controls using ELISA

	ELISA (+)		ELISA(-)	
	Cases	**Percentage**	**Cases**	**Percentage**
Cases (n=60)	19	31.6	41	68.33
Controls (n=60)	4	6	56	93.33
Total	23		97	

P value <0.001 (Statistically Significant)

Among 60 cases with BOH, 19(31.6%) tested positive for ANA by ELISA and 41(68.33%) were ANA negative. Of the total 60 multigravid women, 4(6%) of them tested positive for ANA by ELISA and the remaining 56(93.33%) were negative. The association between ANA positivity,for women with BOH and controls were considered to be statistically significant.(fig 13).

Fig – 13

Evaluation of seropositivity among BOH cases and controls using ELISA

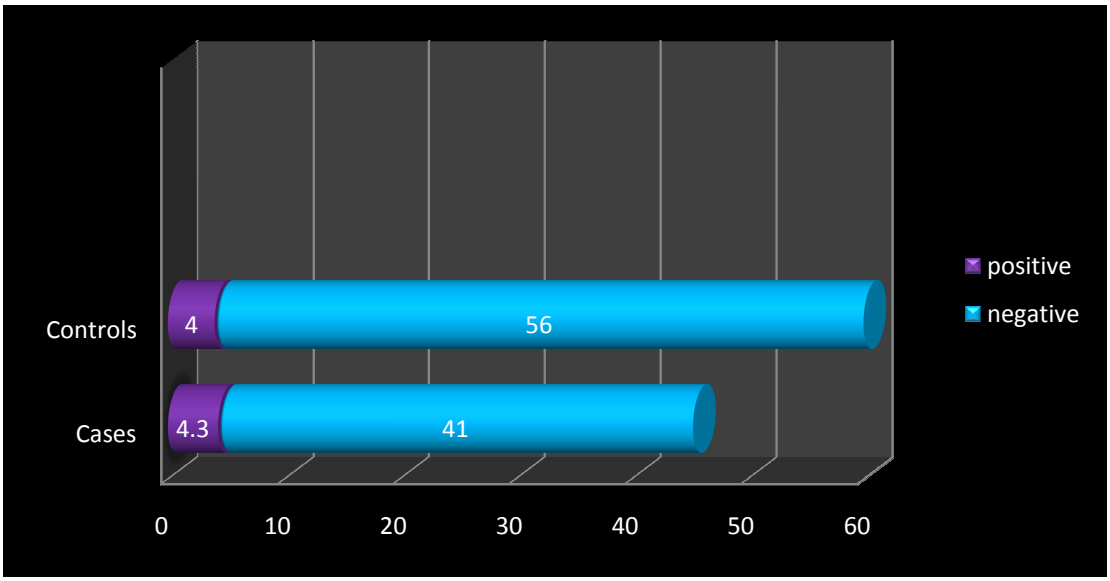


Table - 12

Comparison of ELISA and IFAT for ANA positivity among cases and controls

	ELISA		IFAT	
	No of positives	**Percentage**	**No of positives**	**Percentage**
Cases(n=60)	19	82.6	18	78.26
Controls (n=60)	4	17.39	5	21.73

P value <0.05 Not significant

This table compares ANA positivity among cases and controls by both ELISA and IFAT method. Among BOH cases 19(82.6%), 18(78.26%) were positive by ELISA and IFAT method repectively. Among controls 4(17.39%) and 5(21.73%) individuals were positive by ELISA and IFAT methods respectively.(fig 14)

ELISA reported one more women with BOH as ANA positive, which IFAT failed to detect.Among controls, ELISA missed one sample as ANA positive while IFAT reported as positive.

Fig – 14

Comparison of ELISA and IFAT among cases and controls

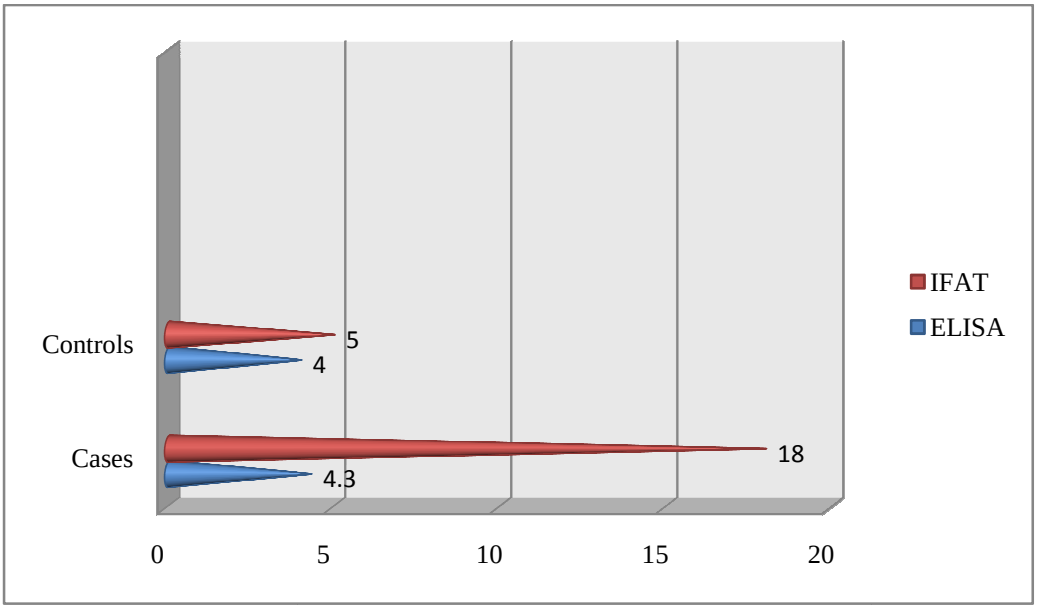


Table - 13

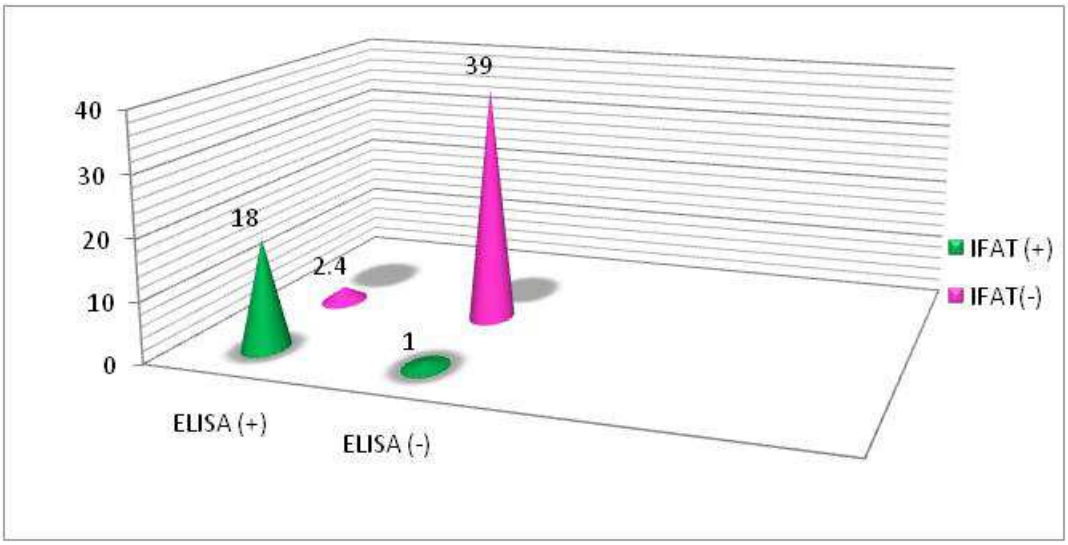
Evaluation of ELISA for detecting ANA positivity among cases with BOH

ELISA	IFAT		Total
	Positive	Negative	
Positive	18	2	20
Negative	1	39	40
Total	19	41	

In the above table Enzyme Immunoassay was evaluated against IFAT.18 cases were positive by ELISA and IFAT.2cases were ELISA positive but negative by IFAT.1 case which was positive by IFAT was negative by ELISA.39 cases were negative by both methods.

Fig - 15

Seropositivity and seronegativity of ANA among cases and controls by ELISA and IFAT



ELISA test was evaluated for its sensitivity and specificity against IFAT, gold-standard test.

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive+False positive}}$$

$$= \frac{18}{19} \times 100 = 94.7\%$$

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative+False positive}}$$

$$= \frac{39}{41} \times 100 = 95.12\%$$

$$\text{Positive predictive value} = \frac{\text{True Positive}}{\text{True Positive+False positive}}$$

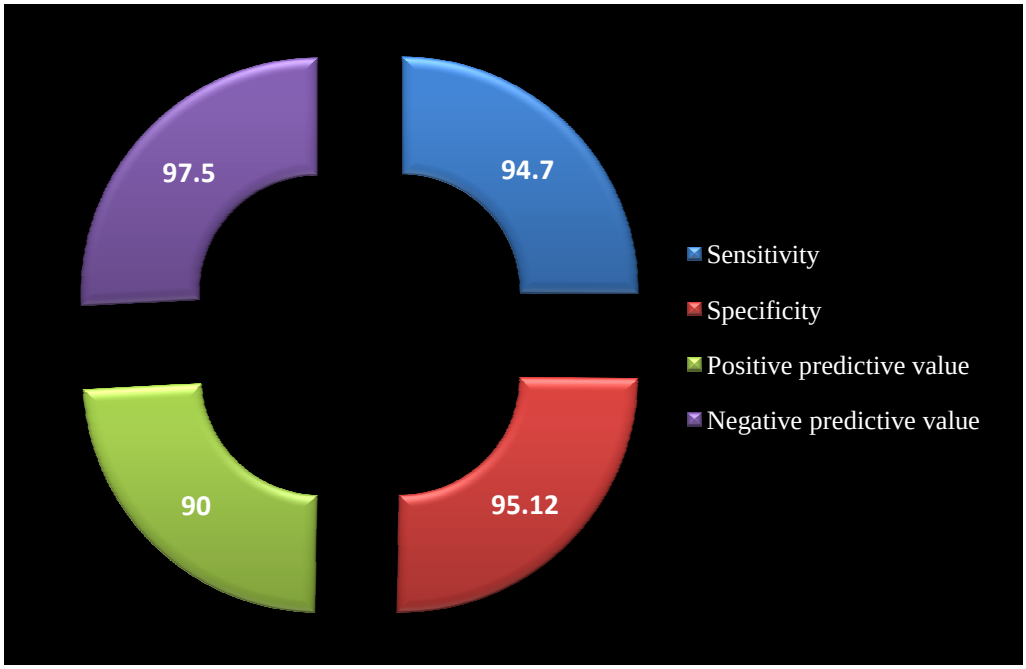
$$= \frac{18}{20} \times 100 = 90\%$$

$$\text{Negative predictive value} = \frac{\text{True Negative}}{\text{True Negative+False Negative}}$$

$$= \frac{39}{40} \times 100 = 97.5\%$$

Fig - 16

Evaluation of ELISA for detecting ANA positivity among cases with BOH



The above table shows sensitivity, specificity, positive predictive value and negative predictive value of ELISA is 94.7%, 95.12%, 90%, 97.5% respectively.

Discussion

6. DISCUSSION

Recurrent pregnancy loss has mutifactorial etiology.Immune factors are believed to play a role in it.Causative relationship between autoantibodies to phospholipids in gestational loss have been extensively studied, but the role of antinuclear antibodies inrecurrent fetal loss is not known.

Indirect immunofluorescence method performed on substrate slides of cultured human epitheloid cell is indispensable in clinical laborataries for the detection of antinuclear antibodies. Enzyme immunoassay (ELISA) kits for antinuclear antibodies detection have been developed and commercialised, but their usefullness have not been studied widely.

This study was conducted on 120 pregnant women in two groups.60 pregnant women with bad obstetric history were evaluated against 60 multiparous pregnant women The study population mainly consisted of women attending antenatal clinic and inpatients in Obstetrics and Gynaecology Department, Tirunelveli Medical College Hospital, from August 2011 to September 2012.

6.1 Age-wise distribution

In this study, the mean age among cases and controls is around 28 years. Patton P E[52] tested sera from 136 women (84 pregnant and 52 nonpregnant) for antibodies directed against nuclear antigens, The mean ages of the two groups were 32 and 26 years, respectively. A study from

Sweden reported the risk of abortion to increase thrice from 8.7% at age 24 to 24.7% at age 35.

6.2 Seropositivity among BOH cases and controls

In this study antinuclear antibodies were found in 18(30%) women with RPL and 5(8.3%) in the control group. In retrospective studies, antinuclear antibodies were found to be five times more common in women with unexplained recurrent miscarriage compared to women with successful pregnancies**.**

In the study conducted by Garcia *et al*[53] antinuclear antibodies were more prevalent in patients with RPL (30%) ,compared to controls (6.6%).Nakatsuka M *et al*[55] in his study reported ANA in 43.5% in women with habitual abortion. and 22.4% in the control group.

A study conducted by Bustos[62] on 118 women with BOH against 125 controls found no difference between controls and patients with antinuclear antibodies.A study conducted by Ruiz JE[63] in Colombian women found no difference in antibodies directed against nuclear proteins in women with recurrent fetal wastage when compared to controls.

6.3 Seropositivity of ANA among normal pregnant women

The occurrence of ANA in normal pregnancy has been variably reported to range from 1-53% .In this study 5(8.3%) women were found to be positive for ANA. Farnam *et al*[50] found an ANA frequency of

10.7% among 214 pregnant women at low titres. Similar findings were reported by Rosenberg, Baig & Shere *et al*, Vazqueg-del Mercado *et al*.

Thus ANA may be positive in low titres in normal pregnancy, not necessarily being an indicator of collagenosis, some infectious disease may be the cause.

6.4 Seropositivity among women with BOH

In this study, of the 60 women with recurrent fetal losses, 18(30%) were found to be positive for antinuclear antibodies. This is comparable to studies conducted by Garcia *et al*[53], Xu L[54] and Nakatsuka *et al*[55].

In the study conducted by Malinowski *et al*[57] the seropositivity of ANA was around 51.5% in women with unexplained RPL, and in the study by Ticconi *et al* it was around 50%.

Antibodies directed against nuclear proteins interfer with the formation and maturation of placenta and may lead to fetal loss.All forms of cells of fetal origin cross placenta, during normal gestation and reach the maternal circulation and tissues, where they remain for long time even after delivery, this fetal microchimerism,[11] may lead to the development of autoantibodies in pregnant women.

6.5 Analysis of risk factors

This study is composed of 42 women with 3rd gravida comprising 44.44% positivity in antinuclear antibodies, but the reason to this seems to be unclear.

Recent articles suggest that the risk of subsequent gestational loss is approximately 24% after two abortions, 30% after three subsequent abortions.In this study, frequency of women presenting with ≤2 abortions was around 30% and >2 abortions was around 50% .

In a study done by Stricker[59] on 47 women, the mean number of abortions was 3.7.

A study in Italy[81] with eighty nine women women with two gestational loss and hundred and five women with three or more gestational loss contributes to 45.87% and 54.12% positivity in the study and concluded that presence of ANA does not distinguishes women with one or more gestational losses.

In the study group[51],1 st trimester abortion occurred in 15 women of which 40% were ANA positive and in 31 women with 2nd trimester abortion , 30% were ANA positive.

Autoimmune diseases are the cause of late 1st trimester or early mid trimester abortions. In pregnancy autoantibodies may react against trophoblast resulting in sub-placental clots which may interfere with

implantation and thereby causing defective placentation. Thrombosis may occur, which results clinically in abortions and fetal wastage[82,83].

In a study conducted by Farnam J [50] to find out total positive cases among 214 antenatal women and in 50 age-adjusted controls , 23 antenatal women were found to have antibodies directed against antigens, five (9.2%) were in the II trimester, and 18 (13.4%) were in the III trimester.

Rosenberg[51] evaluated early, mid, late gestational samples from 100 women At serum dilutions of 1 in 80, the numbers of samples positive in the early, mid, late gestations and cord sera were 18, 21, 21, and 15, respectively. At serum dilutions of 1 in 160, the numbers of sera positive for antinuclear antibodies in each trimester and in cord sera were 9, 12.

Bahar *et al*[56] conducted a controlled study on 103 patients with unexplained recurrent abortions. No difference was found between first and second trimester aborters.

In this study co-morbid conditions like hypothyroidism, HT and gestational diabetes mellitus were present in 16 women of which 10(62.5%), were found to have antinuclear antibodies.The most common association was with hypothyroidism. These women, would be positive for one or more autoantibodies to thyroglobulin or thyroid peroxidase. All

these conditions predispose to vasculitis and leads to sub-placental thrombosis.

A study conducted by Ticconi *et al*[81] in Italy, suggest that (53.6%) of females with recurrent fetal wastage,had multiple autoantibodies along with ANA.

6.6 Analysis of immunofluorescence and ELISA for detection of ANA

In this study 23 women were positive for antinuclear antibodies including 18(30%) of women with BOH and 5(8.3%) controls by IFAT using human epithelioma cell type2 as substrate. Similar findings are reported by Garcia *et al*, Xu L.

In a study done by Ogaswarsa[84] in America, 301 women with unexplained miscarriage, antibodies directed against nuclear antigens was present in 25.2% of study population.

Among the nuclear patterns, the most frequent consist of homogeneous/peripheral fluorescence (DNA, deoxyribonucleoprotein, histones) and speckled fluorescence (RNP,Sm, Ro/SSA, La/SSB); the relatively frequent patterns include the centromeric (CENP-A, CENP-B, CENP-C), Cytoplasmic patterns are relatively less frequent[22].

This study revealed homogenous pattern in 8(44.44%), followed by speckled and anti-centromere pattern in 7(38.8%),6 (26.08%) respectively. Cytoplasmic pattern was seen in 1(4.65%) individual . This

is similar to findings of Satoh *et al*[35] in USA, Edwards *et al*[36] in Britain and Afemann[68] in South Africa.

In India Chopra *et al*[67] detected ANA by Immuno-enzyme method using filter paper blood clots in 21 patients with proven autoimmune disease and reported homogenous pattern in 11 individuals followed by speckled pattern in 10 individuals.

A study conducted by Marin[49] in normal population and Maddison in Spain revealed speckled pattern in almost 50.3% individuals and the other patterns were less frequent

In this study, 3(+) intensity was noted among 5(21.7%) ,2(+) among 6(26%), 1(+) among 11(47.8%) and borderline seen in 1(4.34%) persons.

This way of expressing result has the advantage of saving time and resources because titration of serum samples is not requested; however, the intensity of fluorescence in the working dilution is not always a proportional to the antibody concentration, particularly for centromeric and nucleolar patterns in which the reduced amount of antigen makes the definition of fluorescence intensity highly subjective and, thus, poorly accurate.Equivocal reports must be confirmed by other methods[23].

In this study among 120 patients, 19(31.8%) of women with BOH and 4(6%) of controls were positive by ELISA. Bahar *et al*[56] from Saudi Arabia, reported 13.6% positivity among cases and 1.2% among controls.

In this study antinuclear antibodies were positive in 18 (30%) cases by IFAT and 19 (31.6%) cases by ELISA. Among controls 5(8.3%), 4(6%) samples were positive by IFAT and ELISA respectively. The ability of test to detect true positives, false positives were 94.7%, 95.12%.Percentage positivity and negativity of Enzyme immunoassay was 90 and 97.5 respectively.

A study conducted in Japan by Kumagai[69] screened 257 healthy subjects between Enzyme Linked Immunosorbent Assays and IFAT. The sensitivity and specificity of Enzyme Linked Immunosorbent Assays were 84% and 94%, respectively.

Divate *et al* (2002),[70] investigated the sera from 96 patients, and found the, ability of test to detect true positives, false positives were 90.7%, 85.7%.Percentage positivity and negativity of Enzyme immunoassay was 89.1%, 87.8% respectively.

In Spain, Gonzalez[71] found no differences between areas under recciever operating characteristic curves for enzyme immunoassay and IFAT.

Dipti[75] in Bangladesh ,tested 40 children with Lupus and found IFAT method to be superior than Enzyme Linked Immunosorbent Assay for detection of antibodies to nuclear antigens.In this test true positive rate by IFAT was 100%, in contrary to Enzyme Linked Immunosorbent Assay which was only 55% positive.

A study conducted in Israel[73], from 85 sera of patients with connective tissue disorders, Enzyme Linked Immunosorbent Assay to be superior than IFAT for diagnosis of antinuclear antibodies.

Despite recent advances in the standardization of the IFAT method (automation of the analytic procedure and recognition of the immunohistochemical pattern by way of computerized systems), this technique has some methodologic and interpretive limitations. A negative finding for ANA on IIF may occur in connective tissue diseases owing to the effective absence of antinuclear autoantibodies, paticularly the presences of antibodies to very soluble antigens (such as Ro/SSA), or the presence of autoantibodies directed against scarce cytoplasmic antigens (such as Jo-1 and Ro/SSA).

IFAT gives low titre values in few healthy persons. Every one in four person will test positive for autoantibodies in lower dilutions[85,86]. Need for skilled microscopist to interpret patterns, high inter-personal varaiation may at times give lower sensitivity when compared to Enzyme assays.

ELISA appears to suite as a preliminary investigatory tool to diagnose autoimmune diseases.This methodology is simple with least subjective variation and could be used to screen to large population at a time.

Equivocal results in Enzyme Linked Immunosorbent Assay are to be interpreted by other specific methods for autoantibodies.Moreover this technique has to be standardised by using samples from community and diseased.

So immunofluorescence method is superior to EIA, because fluorescent patterns which have clinical implications would be visualised only by this methodology[4,70].

The ideal method should fulfill criteria of clinical sensitivity and high specificity, precision and accuracy, ease of execution, limited use of technology, quick availability, and contained costs. At present, no method exists that fulfills all these requirements[22].

Western blot[4,22] is a qualitative method that furnishes a complete pattern of the antinuclear specific antibodies and indicates the composition and molecular weight of the different antigenic systems, but the proteins used as antigens are modified in their conformation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

This method is used mostly to confirm autoantibody specificity and to attempt recognizing antigens different from those listed in the preceding guideline.

Thus women without an autoimmune disorder history, but with complications during pregnancy, have a raised number of autoantibodies. This may be due to exacerbation of an undiagnosed autoimmune disorder

or an unknown maternal immune reaction. It is not known whether the occurrence of autoantibodies, is transient and limited to pregnancy and immediate postpartum period, or it is predictive of future immunological disease.

Summary

7. SUMMARY

This study was conducted for a period of 1 year from August 2011 to September 2012. The study population mainly consisted of women attending antenatal clinic and inpatients in Obstetrics and Gynaecology Department. Tirunelveli Medical College Hospital, Tirunelveli.

- A total of One hundred and twenty blood samples collected from patients in two groups. One group consisted of 60 women with Bad Obstetric History.The other group consisted of 60 multiparous women from the age 18-40 years.
- The mean age among cases and controls is 28 years.
- Out of 120 patients tested,18 (30%) women with bad obstetric history and 5(8.3%) women among control group were found to be positive for antinuclear antibodies
- The mean age for women with Bad Obstetric History was 28 years. Of the 21 (35%) women tested in 26-30years, 8(44.44%) women were positive.
- Of the 60 women in study group, women with 3rd gravida constitutes 42%, of them 8 (44.4%) women were positive for antinuclear antibodies.
- There was no statistical difference between seropositivity of ANA and number of abortions.

- There was no statistical difference in antinuclear antibodies based on trimester of pregnancy. Late 1st trimester or early 2nd trimester pregnancy loss could be due to autoimmunity.
- Among the 60 women in study group, co-morbid conditions like hypothyroidism, hypertension, diabetes mellitus were observed in 16(26.6%) women, of which 10 (62.5%) were found to have antinuclear antibodies.There was significant statistical difference between ANA positivity and co-morbid conditions.
- Of the total samples tested, 18(30%) women in study group and 5(8.3%) women in control group were found to be positive for antinuclear antibodies by Indirect Immunofluorescence using HEp-2 cell substrate.
- The most frequent HEp-2 cell pattern observed in the study was homogenous in 8(34.7%) followed by anti-centromere in 6(26.08%). The other patterns observed were speckled, vimentin and nuclear rim.
- Among 23 women, found to be positive for antinuclear antibodies, 1(+) grading is seen in 11(47.8%) samples, 2(+) grading seen in 6(26%) samples, 3(+) grading seen in 5(21.7%) samples.
- Of the total samples tested, 19 (31.6%) women in study group and 4 (6%) women in control group were found to be positive for antinuclear antibodies by ELISA.

- The sensitivity and specificity of ELISA was 94.7% and 95.12% respectively.The positive predictive value was 90% and negative predictive value is 97.5%.
- There was no significant difference in detection of antinuclear antibodies by ELISA and Immunofluorescence among cases and controls.

Conclusion

8. CONCLUSION

- The present study shows that autoantibodies play a role in recurrent pregnancy loss; and patients with bad obstetric history should be evaluated for antinuclear antibodies
- Since abortions due to immune causes occurs in late 1st trimester or early mid-trimester, patients should be screened as early as possible.
- Women who presents with hypothyroidism, hypertension, diabetes mellitus in pregnancy tend to have an associated antinuclear antibody, so these patients also to be evaluated.
- Normal pregnant women have autoantibodies, probably due to some cross-infections which interferes with test result.
- Indirect Immunofluorescence is superior to identify antinuclear antibodies directed against nuclear antigens.
- ELISA appears to be a screening procedure to rule out negative samples.It is easy to perform, accurate, with least interpersonal variations and can be used to screen large population.
- Samples with borderline results are to be confirmed by other methods.

- Immunofluorescence is superior, because it can identify different patterns, which are specific for certain diseases, which is not visualised by Enzyme Linked Immunosorbent Assay.

ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN PREGNANT WOMEN WITH BAD OBSTETRIC HISTORY
MASTER CHART

S.no	age	spouse age	gravida	abortion	trimester	spont/induced	vaginal delivery	LSCS	sys illness	treat his	contaception -any	VDRL test	TORCH panel	IFAT res	IFAT pattern	ELISA res	OD Value
1	29	33	4	3	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.465
2	34	37	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.488
3	31	36	6	4	1	spont	Nil	na	DM- 3yrs	insulin	nil	non-reactive	negative	neg		neg	0.605
4	27	32	4	3	2	spont	Nil	na	nil	nil	nil	non-reactive	positive	neg		neg	0.396
5	23	28	3	2	2	spont	Nil	na	nil	nil	nil	reactive	negative	neg		neg	0.883
6	29	34	4	3	1	spont	Nil	na	HT	nil	nil	non-reactive	negative	pos	fine speckled	pos	3.2
7	25	36	4	3	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.489
8	18	22	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	homogenous	pos	2.24
9	33	38	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.544
10	37	38	4	2	1	induced	Nil	na	nil	nil	nil	non-reactive	positive	neg		neg	0.296
11	25	28	3	2	3	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.258
12	27	30	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.239
13	21	28	3	2	3	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.342
14	25	29	4	2	1	spont	Nil	na	hypothroidism-10yrs	L-thyroxine	nil	non-reactive	negative	pos	fine speckled	pos	4.41
15	37	40	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.349
16	21	27	3	3	2	spont	Nil	na	nil	nil	nil	non-reactive	positive	neg		neg	0.816
17	22	27	3	2	1	spont	Nil	na	nil	nil	nil	reactive	negative	neg		neg	0.204
18	19	24	3	3	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.219
19	26	30	3	2	2	spont	Nil	na	DM- 20 yrs	insulin	nil	non-reactive	negative	pos	coarse speckled	pos	2.38
20	33	37	4	2	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.495
21	35	39	3	2	2	spont	Nil	na	HT-4yrs	insulin	nil	non-reactive	negative	neg		neg	0.233
22	27	31	4	2	2	spont	Nil	na	nil	nil	nil	non-reactive	positive	neg		neg	0.134
23	19	28	3	2	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.258
24	26	30	3	2	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.243

ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN PREGNANT WOMEN WITH BAD OBSTETRIC HISTORY

MASTER CHART

S.no	age	spouse age	gravida	abortion	trimester	spont/induced	vaginal delivery	LSCS	sys illness	treat his	contaception -any	VDRL test	TORCH panel	IFAT res	IFAT pattern	ELISA res	OD Value
25	27	31	3	2	2	spont	Nil	na	nil	nil	nil	reactive	positive	neg		neg	0.329
26	36	40	3	2	2	spont	Nil	na	nil	nil	nil	reactive	negative	neg		neg	0.463
27	34	38	3	2	2	induced	Nil	na	hypothroidism-10yrs	L-thyroxine	nil	non-reactive	negative	neg		neg	0.269
28	28	32	3	2	1	induced	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.244
29	36	39	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.574
30	29	33	4	3	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	homogenous	pos	1.323
31	25	28	5	3	2	spont	Nil	na	hypothyroidism- 4 yrs	L-thyroxine	nil	non-reactive	negative	pos	homogenous	pos	4.42
32	19	27	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.366
33	28	33	3	2	3	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.912
34	32	35	3	2	2	spont	Nil	na	HT-4yrs	nil	nil	non-reactive	positive	pos	nuclear rim- B	neg	0.433
35	36	39	3	2	2	spont	Nil	na	DM, HT-6yrs	insulin	nil	non-reactive	negative	neg		neg	0.416
36	30	36	3	2	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.774
37	20	27	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.877
38	27	31	3	2	3	induced	Nil	na	DM-25yrs	insulin	nil	non-reactive	negative	neg		neg	0.568
39	19	27	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.231
40	33	38	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	fine speckled	pos	1.22
41	25	26	3	2	3	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.312
42	26	31	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.289
43	20	28	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	positive	neg		neg	0.42
44	38	42	3	2	1	spont	Nil	na	HT-4yrs	nil	nil	non-reactive	negative	neg		neg	0.561
45	25	26	3	2	2	spont	Nil	na	nil	nil	nil	reactive	negative	neg		neg	0.501
46	21	26	4	3	1	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		pos	2.9
47	30	33	4	3	3	spont	Nil	na	DM, HT-6yrs	nil	nil	non-reactive	negative	pos	anti-centromere	pos	4.8
48	28	34	4	2	2	induced	Nil	na	nil	nil	nil	non-reactive	negative	pos	anti-centromere	pos	3.86
49	26	36	3	2	2	induced	Nil	na	MS- since childhood	nil	nil	non-reactive	negative	neg		pos	4.12

ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN PREGNANT WOMEN WITH BAD OBSTETRIC HISTORY

MASTER CHART

S.no	age	spouse age	gravida	abortion	trimester	spont/induced	vaginal delivery	LSCS	sys illness	treat his	contaception -any	VDRL test	TORCH panel	IFAT res	IFAT pattern	ELISA res	OD Value
50	29	37	3	2	1	spont	Nil	na	hypothroidism-4 yrs	L-thyroxine	nil	non-reactive	negative	pos	coarse speckled	pos	3.5
51	34	35	5	3	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	homogenous	pos	5.613
52	31	37	6	4	3	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	homogenous	pos	5.11
53	24	28	3	2	2	spont	Nil	na	hypothyroidism -5yrs	L-thyroxine	nil	non-reactive	negative	pos	anti-centromere	pos	3.9
54	27	30	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	anti-centromere	pos	5.32
55	30	36	3	2	2	spont	Nil	na	nil	nil	nil	reactive	negative	neg		neg	0.89
56	34	39	3	2	1	spont	Nil	na	hypothyroidism- 5 yrs	L-thyroxine	nil	non-reactive	negative	pos	homogenous	pos	5.213
57	21	27	3	2	2	induced	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.882
58	37	40	3	2	2	spont	Nil	na	nil	nil	nil	non-reactive	negative	neg		neg	0.662
59	29	32	4	2	3	spont	Nil	na	nil	nil	nil	non-reactive	negative	pos	homogenous	pos	4.78
60	31	38	4	3	1	spont	Nil	na	hypothyroidism- 3 yrs	L-thyroxine	nil	non-reactive	negative	pos	anti-centromere	pos	1.99
C1	26	29	3	nil	3	nil	yes	na	nil	nil	yes	non-reactive	not applicable	pos	homogenous	neg	1.23
c2	28	37	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.651
c3	32	33	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.307
c4	20	26	2	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.901
c5	21	26	3	nil	3	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.262
c6	27	31	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.612
c7	30	34	4	nil	3	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.424
c8	25	30	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.513
c9	26	31	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.555
c10	36	39	4	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.346
c11	24	26	3	nil	2	nil	yes	na	nil	nil	yes	non-reactive	not applicable	neg		neg	0.502
c12	31	36	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		pos	2.29
c13	35	39	3	nil	2	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	pos	vimentin pattern	neg	0.426
c14	28	32	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.638

ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN PREGNANT WOMEN WITH BAD OBSTETRIC HISTORY
MASTER CHART

S.no	age	spouse age	gravida	abortion	trimester	spont/induced	vaginal delivery	LSCS	sys illness	treat his	contaception -any	VDRL test	TORCH panel	IFAT res	IFAT pattern	ELISA res	OD Value

c15	25	37	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.393
c16	29	33	3	nil	1	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.709
c17	20	27	2	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.728
c18	37	41	4	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.392
c19	21	25	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.441
c20	19	24	2	nil	2	nil	yes	na	nil	nil	yes	non-reactive	not applicable	neg		pos	2.11
c21	27	31	2	nil	3	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.342
c22	36	40	2	nil	3	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.29
c23	30	33	4	nil	2	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	pos	coarse speckled	pos	1.99
c24	24	28	3	nil	2	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.311
c25	27	31	4	nil	3	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.342
c26	26	32	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.211
c27	22	26	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.39
c28	31	36	3	nil	2	nil	yes	na	nil	nil	yes	non-reactive	not applicable	neg		neg	0.611
c29	21	25	3	nil	2	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.917
c30	28	32	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.342
c31	29	40	3	nil	3	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.267
c32	34	38	5	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.438
c33	24	27	2	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.511
c34	37	41	3	nil	3	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.574
c35	30	36	4	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.325
c36	26	31	3	nil	2	nil	not applicable	yes	nil	nil	yes	non-reactive	not applicable	neg		neg	0.388
c37	28	34	2	nil	2	nil	yes	na	nil	nil	yes	non-reactive	not applicable	neg		neg	0.861
c38	30	34	2	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.79

ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN PREGNANT WOMEN WITH BAD OBSTETRIC HISTORY
MASTER CHART

S.no	age	spouse age	gravida	abortion	trimester	spont/induced	vaginal delivery	LSCS	sys illness	treat his	contaception -any	VDRL test	TORCH panel	IFAT res	IFAT pattern	ELISA res	OD Value

c39	32	36	2	nil	2	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.813
c40	35	40	3	nil	3	nil	yes	na	nil	nil	nil	non-reactive	not applicable	pos	anti-centromere	pos	1.89
c41	26	31	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.631
c42	19	26	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.622
c43	36	41	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.421
c44	32	36	3	nil	3	nil	yes	na	nil	nil	yes	non-reactive	not applicable	neg		neg	0.712
c45	37	41	2	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.842
c46	26	26	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.608
c47	20	28	2	nil	2	nil	not applicable	yes	nil	nil	yes	non-reactive	not applicable	neg		neg	0.419
c48	34	38	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	pos	fine speckled	neg	0.222
c49	32	33	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.602
c50	28	35	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.256
c51	24	30	2	nil	2	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.326
c52	21	26	3	nil	1	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.356
c53	21	28	2	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.234
c54	34	37	3	nil	3	nil	not applicable	yes	nil	nil	yes	non-reactive	not applicable	neg		neg	0.632
c55	30	34	2	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.289
c56	30	37	3	nil	1	nil	yes	na	nil	nil	yes	non-reactive	not applicable	neg		neg	0.534
c57	24	26	2	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.328
c58	27	31	3	nil	2	nil	yes	na	nil	nil	nil	non-reactive	not applicable	neg		neg	0.811
c59	22	27	3	nil	1	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.558
c60	35	40	3	nil	3	nil	not applicable	yes	nil	nil	nil	non-reactive	not applicable	neg		neg	0.431

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Annexure 1

ANNEXURE I

DATA SHEET

For P.G Dissertation Work on

Association of Antinuclear Antibodies on Pregnant Women with Bad Obstetric History

I.General information

Name: Age:

Spouse age:

Address Occupation:

OP/IP No: IP- Unit/ward:

Income: Urban/Rural:

Educational status: Occupation of partner:

II Antenatal risk assessment

Menstrual history:

LMP: Gravida:

EDD: Para:

CEDD: Live:

Abortion/still birth: MTP:

LSCS: LCB:

Previous obstetric history:

Status of baby born:

III Clinical assessment

Presenting complaints

Bleeding per vaginam:

Lower abdominal pain:

Expulsion of fleshy mass per vaginam:

Vaginal discharge:

Systemic illness

H/O diabetes mellitus:

H/O hypertension:

H/O tuberculosis:

H/O sexually transmitted diseases:

H/O contraceptive usage:

H/O drug intake Duration:

Family history of similar illness:

Habits: Smoking/ Tobacco/Alcohol

IV Clinical Diagnosis:

V Treatment/advised given in unit:

VI Follow up of patient(if needed):

VII Laboratory investigations:

Complete blood count

Total count: ESR:

Differential count: Hb:

Blood Urine

Sugar : Albumin:

Urea : Sugar:

Creatinine: Deposits:

Blood group:

VDRL:

HIV status:

Thyroid function test:

TORCH panel

USG:

ANA:

IFAT:

Grading:

Pattern:

ELISA:

Date:

Signature:

Annexure 2

ABBREVIATIONS

ANA	-	Antinuclear Antibodies
BOH	-	Bad Obstetric History
RPL	-	Recurrent Pregnancy Loss
ENA	-	Extractable Nuclear Antigen
CLIF	-	Crithidia Luciliae Immunofluorescence
Farr assay	-	Radiol-labelled assay
MIA	-	Multiplex Immunoassay
HEp-2 cell	-	Human Epithelioma type-2 cell
ANCA	-	Antineutrophilic Cytoplasmic Antigen
FPBC	-	Filtered Paper Blood Clots
IVIg	-	Intravenous Immunoglobulin therapy
FITC	-	Fluorescein isothiocyanate
TMB	-	3,3’,5,5’-tetramethyl benzidine

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Institutional Ethical Committee

Certificate of Approval

This is to certify that the Institutional Ethical Committee of this College unanimously approves the Thesis /Dissertation/ Research Proposal submitted before this committee by Dr. A.ANUPRIYA , a **POST GRADUATE STUDENT IN THE DEPARTMENT OF MICROBIOLOGY** in the Department of **MICROBIOLOGY**, of Tirunelveli Medical College /Hospital, Tirunelveli titled **"ASSOCIATION OF ANTINUCLEAR ANTIBODIES IN WOMEN WITH BAD OBSTETRIC HISTORY "** registered by the IEC as 156/G.S/IEC/2011 dated. 22.03.2012. The Investigator is hereby advised to adhere to all the stipulated norms and conditions of this ethical committee.

Issued on this Date

22.03.2012

Under Seal

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Secretary

Secretary,
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